

**THE DEVELOPMENT OF PORCINE
PREANTRAL FOLLICLES *IN VITRO***

Monica Clare Bark

Ph.D.

University of Edinburgh

2004



Abstract

The development of a culture system to sustain early ovarian follicle growth to a stage where live offspring can be obtained has thus far only been achieved in the mouse (Eppig and Schroeder, 1989; Spears et al, 1994; Eppig and O'Brien, 1996; Cortvrindt et al, 1998; O'Brien et al, 2003). It is unlikely that this will be achieved in the domestic species in the near future; however *in vitro* culture systems provide a useful tool for the study of early follicle development and identification of markers of development. The use of a serum-free system is useful to analyse the contents of medium, and specific effects of factors added to medium can be monitored.

The objectives of this thesis were (1) to compare different culture systems *in vitro* on porcine preantral follicle development, and to analyse the effects of adding specific factors to serum-free medium, and (2) identify possible markers of development of pig oocyte and follicle development. Follicles were cultured using two systems; individually in McCoys medium to identify the effects of ascorbate, FSH, and serum, and secondly in NCSU medium individually and in-groups to identify the effects of group and individual culture. Results from the McCoys experiment revealed that somatic cell death was significantly reduced in follicles cultured in the presence of ascorbate in comparison with other treatment groups, but the vitamin was found to have no effect on follicle growth. Follicle growth was significantly enhanced by the addition of FSH to serum-free medium, but had no effect on cell death of follicles. Culture in NCSU medium revealed that follicles

grew best when cultured individually in the presence of serum in 96-well plates in comparison to follicles cultured in-groups in 24-well plates.

The identification of markers of development for follicles and oocytes would aid the development of a culture system for preantral follicles. Markers of development could be used to give an indication of follicle developmental stage, of oocyte maturational stage, or potentially as an indicator of follicle or oocyte health.

Growth and Differentiation Factor-9 (GDF-9) is a possible indicator of follicle and oocyte developmental stage. It has been identified in several species, including human, rodents, cattle and sheep, but not in the pig. In this study the exon 2 portion of GDF-9 was isolated using human and mouse primers, and sequenced. It was found to display 88% homology to the human sequence. RT-PCR revealed that it was expressed strongly in the porcine oocyte, but not in granulosa, skin or intestinal cells. Bone Morphogenetic Protein-15 (BMP-15) also appeared to be oocyte-specific in the pig, using sheep primers and RT-PCR to identify its location.

Matrix metalloproteinase-9 (MMP-9) has been identified as a marker of follicle health in *in vitro* grown follicles in the bovine (McCaffery et al, 2000). In the present study cultured porcine preantral follicles were found to produce MMP-2 and MMP-9 on days 2, 4 and 6 of culture. However, there was no correlation between the production of these proteases and any other factor, e.g. somatic cell health. It was found, however, that oocytes that were cultured when stripped of their surrounding somatic cells appeared to produce more MMP-9 than those cultured intact in oocyte-cumulus cell complexes; however, this difference was not

significant. Correspondingly, oocytes cultured in complexes produced significantly more MMP-2 than those cultured without their companion somatic cells.

In conclusion, the main findings of this thesis are that ascorbate has a positive effect on porcine preantral follicles by reducing granulosa cell death *in vitro*. FSH has a stimulatory effect on the growth of preantral follicles, but was not found to be a survival factor in this system. The mature peptide of porcine GDF-9 was isolated and sequenced, and was found to be 88% homologous to the corresponding human sequence. It was found to be strongly expressed in the porcine oocyte, as was BMP-15. MMP-2 and MMP-9 were found to be secreted by porcine preantral follicles and oocytes *in vitro*. Although no significant differences were found in the secretion patterns of cultured follicles *in vitro* according to treatment or health or developmental status of follicle, oocytes seemed to produce more MMP-9 when cultured in the absence of cumulus cells, and more MMP-2 was produced when cumulus cells are present.

Publications arising from this thesis:

Abstracts of Oral Presentations

Bark MC and Telfer EE. Effects of Follicle Stimulating Hormone and Ascorbic Acid on Porcine Preantral Follicle Development *in Vitro*. Proceedings of the 2002 National Ovarian Workshop, Fitzwilliam College, Cambridge, 25-26 March. Abstract16.

MC Bark, CJH Souza, C MacDougall & EE Telfer. The isolation and location of the oocyte-specific factor GDF-9 in the porcine ovary. Reproduction 2002; Abstract Series 29:1.

Acknowledgements

Firstly I would like to thank my supervisor Dr Evelyn Telfer for all her support, encouragement and guidance through the duration of this PhD. I would also like to thank everyone who I have worked with during the PhD, including John Binnie for help with dissection and histology, and Debbie Bruce, Kirsty Walters and Fiona Thomas for advice and technical support. Colin MacDougall, Sharon White and Carlos Souza were extremely kind in giving me technical advice on molecular techniques, especially thanks to Colin for getting me starting on the GDF-9 experiments.

I would also like to express extreme gratitude to Dr Simon Riley for allowing me into his lab to run samples for MMP analysis, and for preparing the zymography figures for the thesis. I would also like to thank Rose Leask for all her help in running the gelatin zymograms, and also for performing some of the zymograms (chapter 5). Everyone's help and advice was greatly appreciated.

I would also like to thank my academic sponsors, BBSRC, and also my industrial sponsors from the Pig Improvement Company (now SYGEN), for their financial assistance, and also for providing some extremely useful training courses, and the annual PIC student conference.

Finally I would like to thank my family for all their support; I couldn't have managed without it, especially Mum and Dad! Thanks again. Also thanks to Joanne, Catriona, Eilidh, Brenda and Mike for their continuous support and encouragement.

Abbreviations

AMH	Anti-mullerian hormone
bFGF	Basic fibroblast growth factor
3 β -HSD	3 β -hydroxysteroid dehydrogenase
17 β -HSD	17 β -hydroxysteroid dehydrogenase
BM	Basement membrane
BMP-15	Bone morphogenetic protein-15
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CG	Chorionic gonadotrophin
cpm	Counts per minute
COC	Cumulus oocyte complex
DEPC	Diethylpyrocarbonate
DHT	Dihydrotestosterone
DPX	p-Xylene-bis (N-pyridinium bromide)
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Foetal bovine serum
FIG- α	Factor in the Germline alpha
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GDF-9	Growth and differentiation factor-9
GnRH	Gonadotrophin releasing hormone
HA	Hyaluronic acid
IGF	Insulin-like growth factor
ISH	In situ hybridisation
IVG	In vitro grown
IVM	In vitro maturation
KL	Kit ligand
LH	Luteinizing hormone
MMP	Matrix metalloproteinase
NaCl	Sodium chloride
NCSU	North Carolina State University Medium
NGF	Nerve growth factor
OOX	Oocyctomized complex
PBS	Phosphate buffered saline
P450 C17	17 α hydroxylase
P450 CSCC	P450 cholesterol side chain cleavage enzyme
PMSG	Pregnant mare serum gonadotrophin
RNA	Ribonucleic acid
SCC	Sodium Citrate
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
SRY	Sex determining region Y
TEA	Triethalone
TGF- α/β	Transforming growth factor- alpha /beta

TNF- α	Tumour necrosis factor-alpha
TIMP	Tissue inhibitor of metalloproteinase
TSH	Thyroid stimulating hormone
WT1	Wilms' tumour-associated gene

Contents Table

Declaration	i
Abstract	ii
Publications	v
Acknowledgements	vi
Abbreviations	vii
Table of Contents	ix
List of Figures	xviii
List of Tables	xx

Chapter 1
General Introduction

Section	Page
1.1 General Introduction	1
1.2 The Oestrous Cycle and Follicle Development	2
1.3 Development of the Gonads and Primordial Follicle Formation	4
1.4 Folliculogenesis	7
-1.4.1 Primordial Follicle Initiation	7
-1.4.2 Follicle Development	8
1.5 Oocyte Development	11
1.6 Oocyte-Follicle Interactions	15
1.7 Hormones and Growth Factors in Folliculogenesis	19
-1.7.1 Pituitary Hormones: FSH and LH	19
-1.7.2 Inhibin and Activin	24
-1.7.3 The IGF System	25
-1.7.4 EGF	25
1.8 Follicular Cell Death	26
-1.8.1 Apoptosis	26
-1.8.2 Cell Death in Ovarian Follicles	26
1.9 Ascorbate	29
1.10 Tissue Remodelling in the Ovary and the MMP System	31
1.11 Follicle Culture Systems	35
-1.11.1 Why Culture Preantral Follicles?	35
-1.11.2 Methods for the Isolation and Culture of Ovarian Follicles	36
-1.11.3 Whole Ovary and Strip Culture	36
-1.11.4 Preantral Follicle Culture	37
-1.11.5 Rodent Systems	40
-1.11.6 Preantral Follicle Culture	41
1.12 Domestic Species Preantral Follicle Culture	42

Chapter 2

General Materials and Methods

Section	Page
2.1 Materials and Methods	49
2.1.1 Preantral Follicle Isolation	49
-2.1.1.1 Collection of Ovaries	49
-2.1.1.2 Dissection Medium	49
-2.1.1.3 Follicle Dissection	49
2.1.2 Preantral Follicle Culture	50
-2.1.2.1 Culture Medium	50
-2.1.2.2 Preantral Follicle Culture	51
2.1.3 Histology	52
-2.1.3.1 Fixation of Tissue	52
-2.1.3.2 Sample Processing	52
-2.1.3.3 Sectioning and Mounting	52
-2.1.3.4 Staining	52
-2.1.3.5 Collection of Histological Results	53

Chapter 3

The Effects of Ascorbate, Follicle Stimulating Hormone and Serum on the Development of Porcine Preantral Follicles *in Vitro*

Section	Page
3.1 Introduction	58
3.2 Materials and Methods	62
-3.2.1 Follicle Isolation and Culture	62
-3.2.2 Treatment Groups	62
-3.2.3 Statistical Analysis	63
3.3 Results	64
-3.3.1 The Effects of Ascorbate, FSH and Serum on Preantral Follicle Growth	64
-3.3.2 The Effects of Ascorbate, FSH and Serum on Follicle Atresia	66
-3.3.3 Histological Examination of Cultured Follicles	68
-3.3.4 Follicle Morphology	70
-3.3.5 The Effects of Ascorbate, FSH and Serum on Cumulus-Oocyte Contact	72
3.4 Discussion	74

Chapter 4

Porcine Preantral Follicle Interactions *in Vitro*, and the Effect of Different Culture Wells and Serum on Development

Section		Page
4.1	Introduction	80
4.2	Materials and Methods	82
	-4.2.1 Collection of Ovaries	82
	-4.2.2 Follicle Dissection	82
	-4.2.3 Follicle Culture Medium	82
	-4.2.4 Statistical Analysis	83
4.3	Results	84
	-4.3.1 Effects of Co-Culture and Serum on Follicle Growth	84
	-4.3.2 Histological Examination of Cultured Follicles	86
4.4	Discussion	90

Chapter 5

The Secretion of the Gelatinases MMP-2 and MMP-9 by Porcine

Follicles and Oocytes *in Vitro*

Section		Page
5.1	Introduction	96
5.2	Materials and Methods	98
	-5.2.1 Preantral Follicle Culture Medium	98
	-5.2.2 Collection of Oocytes and Culture of Cumulus-Oocyte Complexes and Denuded Oocytes	98
	-5.2.3 Zymography and Detection of MMPs in Follicle and Oocyte Conditioned Medium	99
	-5.2.4 Data and Statistical Analysis	100
5.3	Results	101
	-5.3.1 Detection of MMP-2 and MMP-9 in Preantral Follicle Conditioned Medium	101
	-5.3.2 Production of MMP-2 and MMP-9 by Cultured Follicles	104
	-5.3.3 Follicle Atresia and MMP-9 Production	105
	-5.3.4 Oocyte and Cumulus-Oocyte-Complex Production of MMP-2 and MMP-9	107
5.4	Discussion	109

Chapter 6

The Sequence and Expression of GDF-9 and the Expression of BMP-15 in the Porcine Prepubertal Ovary

Section

6.1	Introduction	118
6.2	Materials and Methods	121
	-6.2.1 RNA Extraction	121
	-6.2.2 cDNA Construction	121
	-6.2.3 Cloning	123
	-6.2.4 Localisation of GDF-9	123
	-6.2.5 <i>In Situ</i> Hybridisation and Localisation of GDF-9	124
	-6.2.5.1 Probe Construction	124
	-6.2.5.2 Slide Preparation	125
	-6.2.5.3 <i>In Situ</i> Hybridisation	126
	-6.2.5.4 Developing	126
6.3	Results	127
	-6.3.1 Sequencing of 277bp GDF-9 Fragment	127
	-6.3.2 GDF-9 Location by RT-PCR	128
	-6.3.3 BMP-15 location by RT-PCR	129
	-6.3.4 <i>In Situ</i> Hybridisation	130
6.4	Discussion	131

Chapter 7: Discussion and Conclusions

Section

7	General Discussion	136
---	--------------------	-----

Chapter 8: References

Section

8	References	141
---	------------	-----

	Appendix 1	170
--	------------	-----

	Appendix 2	171
--	------------	-----

	Appendix 3	172
--	------------	-----

List of Figures

Chapter 1

Figure		Page
1.1	Follicle Development	10
1.2	Oocyte Meiosis Resumption	12
1.3	Oocyte-Follicle Interactions	18
1.4	2-cell 2-gonadaotrophin Mechanism	23
1.5	Structure of the Gelatinase MMPs	32
1.6	Culture Systems	39

Chapter 2

Figure		
2.1	Freshly Isolated Follicles	55
2.2	Freshly Isolated Follicle Fixed for Histology	56

Chapter 3

Figure		
3.3.1	Effects of Ascorbate, FSH and Serum on Preantral Follicle Growth	65
3.3.2	Effects of Ascorbate, FSH and Serum on Follicle Atresia	67
3.3.3 a,b	Follicle Morphology	70
3.3.3 c,d	Follicle Morphology	71
3.3.4	Effect of Ascorbate, FSH and Serum on Cumulus-Oocyte Contact	73

Chapter 4

Figure

4.3.1	Effects of Serum and Co-culture on Follicle Growth	85
4.3.2	Effects of Serum and Co-culture on Follicle Atresia	87
4.3.3	Effects of Serum and Co-culture on Oocyte Size	88

Chapter 5

Figure

5.3.1.1	Percentage of Follicles Producing MMP-2	102
5.3.1.2	Percentage of Follicles Producing MMP-9	103
5.3.2	Production of MMP-2 and MMP-9 by Cultured Follicles	104
5.3.3	Percentage of Healthy and Atretic Follicles Producing MMP-9	106
5.3.4	Zymogram of Production of MMP-2 and MMP-9 by Cultured Oocytes and Cumulus-Oocyte Complexes	107
5.3.5	Latent MMP-2 Densitometry Readings of Denuded Oocytes and Cumulus-Oocyte Complexes	108

Chapter 6

Figure		Page
6.3.1	Comparison of Porcine and Human GDF-9 Exon 2 Sequences	127
6.3.2	Expression of GDF-9 in Porcine Oocyte, Granulosa and Intestine Samples	128
6.3.3	Expression of BMP-15 in Porcine Oocyte, Granulosa and Intestine Samples	129
6.3.4	In Situ Hybridisation for GDF-9 in Porcine Antral Follicle	130

List of Tables

Page	Table
69	Table 3.3.1: The effects of ascorbate, FSH and serum on follicle histology
89	Table 4.3.1: The effects of co-culture and serum on follicle histology

1.1 Chapter 1: General Introduction

The main limiting factor in the advancement of current reproductive technologies is the lack of availability of quality fertilisable eggs. There have been significant advances in the development of techniques such as *in vitro* fertilisation, *in vitro* maturation and cloning over the past two decades, but success rates using these procedures are relatively low (Abeydeera et al, 1998; Rideout et al, 2001). The oocytes which are currently used for these procedures are taken from follicles at the antral stage of development. Antral follicles are not an ideal starting population for these *in vitro* techniques as they are highly heterogeneous and have a relatively large incidence of cell death (Guthrie & Garrett, 2001). The prepubertal ovary has an abundant store of early germ cells which could be an ideal starting population of follicles and oocytes due to high levels of homogeneity and low levels of cell death (Erickson, 1967; Faddy et al, 1987). However, the oocytes from these follicles cannot be used for *in vitro* maturation (IVM) as they are incapable of successfully resuming meiosis as they are underdeveloped (Hirao et al, 1994; Sirard, 2001). To harvest the oocytes from these early follicles they would have to be removed from the ovary and grown *in vitro*. An alternative has been suggested to provide a more homogeneous population of oocytes for *in vitro* maturation systems than those which are currently available; *in vitro* grown (IVG) follicles. This technique involves harvesting primordial and preantral follicles, and growing them in *in vitro* culture systems. However, as yet, live young have only been produced in the mouse using these systems with a starting population of primordial follicles (Eppig and O'Brien, 1996; O'Brien et al, 2003), or preantral follicles (Eppig and Schroeder, 1989; Spears et al, 1994; Cortvrindt et al, 1998).

1.2 The Oestrous Cycle and Follicle Development

The domestic pig is a polytocous, non-seasonal breeder. Oestrous cycles in the pig occur about every twenty-one days, and during each cycle follicles are recruited to grow and develop. The process of follicular development takes around four months *in vivo* in the pig, from the primordial stage right through to pre-ovulatory (Morbeck et al, 1992). Around 50 follicles develop during the luteal and early follicular phases, with around 10-20 developing from these follicles to the preovulatory stage. The number of ovulated eggs is normally around 10-20, although this number is affected by factors such as breed of pig (McCoard et al, 2003) and state of nutrition (Prunier and Quesnel, 2000). The oestrous cycle can be divided into stages, known as diestrus (14 days), proestrus (2 days), oestrus (2-3 days), and metestrus. Ovulation occurs at mid-oestrus, and the follicular remnants then luteinize, and form a progesterone producing corpus luteum (see figure 1.1 for oestrous cycle in relation to follicle development).

Although the pig is a non-seasonal, polytocous breeder, with no specific problems in breeding *per se*, *in vitro* grown oocytes could provide quality eggs for use in techniques such as *in vitro* maturation and fertilisation, cloning, and eventually xenotransplantation. Using early follicles could help to improve the success rates of such techniques. Furthermore, since less than 1% of eggs ever ovulate, this represents a tremendous waste of potentially valuable genetic material (Hsueh et al, 1994; Kaipia and Hsueh, 1997). If this material could be harvested from, for example, a genetically valuable sow, this could improve profits greatly from one sow, and potentially improve breeding lines, leading to better quality animals. This technique also has the potential to conserve rare

breeds, and eventually systems developed for animals could conceivably be used to overcome human fertility problems.

One of the main problems limiting the advancement of *in vitro* grown oocyte techniques is a basic lack of understanding of the processes which occur during early folliculogenesis. There are many requirements for follicle development at all stages, and hormones and growth factors act together to control this. In order to develop a culture system capable of supporting oocyte and follicle development through to a stage where the oocyte can be fertilised *in vitro*, a better understanding of early follicle development must be obtained.

The main aim of this thesis was to investigate the processes and factors affecting early folliculogenesis *in vitro*. A better understanding of early processes in follicle development could potentially allow the complete *in vitro* development of primordial follicles from the domestic species and humans to a stage where the oocytes could be removed and fertilised *in vitro*, with the production of live offspring, as has been achieved in the mouse (Eppig and O'Brien, 1996; O'Brien et al, 2003). This is unlikely to become a reality in the domestic species and humans for the foreseeable future, and the use of culture systems in these species is limited to being a valuable experimental tool to study follicle development.

1.3 Development of the Gonads & Primordial Follicle

Formation

The primordial follicle is the functional unit of the mammalian ovary. A primordial follicle consists of an oocyte which is arrested at the dictyate stage of prophase I, and a layer of flattened precursor somatic cells. Primordial follicle formation arises through the development of the mammalian gonads. In the foetus gonads develop on each side of the body from intermediate mesoderm lining the body cavity on the urogenital ridge. Primordial germ cells originate from outside the area of the presumptive gonad. Germ cells migrate through the gut endoderm by amoeboid movement and onto the gonadal ridges (Mintz and Russell, 1957). At this stage the gonads are indifferent. Testes are formed from the indifferent gonad when the single copy gene SRY (sex determining region Y), which is located in the 1A1 region of the Y chromosome, is present in males (Page et al, 1990). In mice it has been shown that XY gonads express the testis determining gene SRY (Koopman et al, 1991), and the presence of this gene initiates the differentiation of the supporting cell precursors as Sertoli instead of the granulosa cells, which would develop in the absence of this gene (Burgoyne et al, 1988; Albrecht and Eicher, 2001). Several other genes have been implicated in male sex development, including the Wilms' tumour-associated gene (WT1). Several natural human mutations of this gene exist, and one such mutation has been observed in heterozygous missense mutations in the zinc finger DNA-binding domain of WT1. This is known as Denys-Drash syndrome, and it can lead to severe and frequent urogenital malformations (Pelletier et al, 1991). These mutant forms of the protein are thought to act as dominant negatives which interfere with wild-type WT1 action. XY individuals with Denys-Drash

syndrome are more affected than XX and they can also display ambiguous or female genitalia, which would suggest that the gene is involved in male urogenital development (Pelletier et al, 1991). Another gene which appears to profoundly influence male gonad differentiation is *sox9*. *Sox9* expression is gonad specific in several species, and mutations of the gene are associated with male-female sex reversals in humans (Kanai and Koopman, 1999). It is therefore apparent that successful differentiation of the gonads into testes is heavily reliant on the inter-play of sex determination genes. Once the gonads begin to develop as testis they secrete factors such as Anti-müllerian hormone (AMH) and testosterone to ensure the rest of the embryo develops accordingly i.e. development of the appropriate secondary sexual characteristics. AMH has been shown to masculinise the gonads by causing Müllerian duct regression (Vigier et al, 1987). Testosterone is secreted by the Leydig cells, which acts on the androgen receptors causing Wolffian duct regression and masculinisation of the genitalia (Josso et al, 2003). Testosterone is then further reduced to dihydrotestosterone (DHT), which acts on the prostate and external genitalia causing masculinisation (Wigley et al, 1994).

Ovary formation occurs when the coelomic epithelium proliferates and begins to invade the intermediate mesoderm to form sex cords. These sex cords degenerate and form separate groups of cells, which become the individual gamete precursors. Primordial follicle formation arises when primitive granulosa cells are organised around the germ cell.

The store of primordial follicles that are present in the mammalian ovary at birth represents the entire germ cell population of the female, and these cells are recruited to grow throughout the reproductive life span of the female. In the

porcine ovary the number of primordial follicles peaks at 50 days after copulation, and at the maximum there are 1.2×10^6 (Erickson, 1967). This number drops sharply after the peak, and at the start of puberty there are some 420,000 germ cells present, representing the entire germ cell population for reproductive life (Gosden and Telfer, 1987). The rest of the follicles have been lost due to apoptosis. At ten days post-partum most of the oocytes are at the diplotene stage of development, and follicle development has already commenced (Black & Erickson, 1968).

1.4 Folliculogenesis

1.4.1 Primordial Follicle Initiation

The recruitment of primordial follicles into the growing pool is a constant process throughout the reproductive life of mammals. Primordial follicles are recruited to grow through an unknown signal, which is thought to be either a relief from inhibition or a stimulatory trigger. During each oestrous cycle primordial follicles are recruited for growth, and join the growing pool of follicles, but it is not known why some follicles remain dormant while others begin to grow. Recruitment into the growing pool is characterised by differentiation of the surrounding somatic cells into cuboidal granulosa cells, and significant oocyte growth. Several candidate molecules for the initiation or repression of primordial follicle growth have been suggested. Anti-mullerian hormone (AMH) has been identified as a candidate molecule for the suppression of primordial follicle initiation (Durlinger et al, 1999; 2002). Knock-out mice for this gene have a significantly larger number of early growing follicles present in the ovary than wild-type mice, and significantly less primordial follicles (Durlinger, 1999). Further studies revealed that mouse ovaries which were cultured in the presence of AMH had significantly less growing follicles than controls (Durlinger et al, 2002). There are several factors which have been identified as potential initiators of follicle growth, including growth and differentiation factor-9 (GDF-9; Vitt et al, 2000), kit-ligand (KL; Parrott & Skinner, 1999), basic fibroblast growth factor (bFGF; Nilsson et al, 2001), and nerve growth factor (NGF; Dissen et al, 2001). GDF-9 has been implicated in the initiation of primordial follicle recruitment, as rats treated with recombinant GDF-9 were found to have decreased primordial follicle numbers, and increased

numbers of growing follicles, indicating GDF-9 had a stimulatory effect on primordial follicle initiation (Vitt et al, 2000). A similar effect was observed when rat ovaries were cultured in the presence of KL, as there were significantly less primordial follicles present in these ovaries after 14 days of KL treatment, and significantly more growing ones (Parrott and Skinner, 1999). Organ culture studies, where ovaries were treated with bFGF displayed a similar pattern of primordial follicle initiation (Nilsson et al, 2001). The neurotrophin NGF has also been implicated in follicle initiation, as mice null for this gene display a marked reduction in the number of follicles recruited to grow in comparison to wild-type and heterozygote mice (Dissen et al, 2001).

1.4.2 Follicle Development

After the primordial follicle has been recruited into the growing pool, it is then classed as a primary follicle (see figure 1.1 for follicle development).

Surrounding granulosa cells differentiate into cuboidal cells, which continue to increase in size and number. Proliferation continues, and the follicle becomes a multi-laminar structure, and is now deemed preantral. At this stage of development the follicle develops a theca layer, which is highly vascularised and steroid producing. The rest of the follicle is highly avascular. The small pools of follicular fluid which are present in the follicle then coalesce to form the antral cavity, and this marks the beginning of the antral stage of development. The follicular fluid contains several components including hormones, growth factors and enzymes (Berne and Levy, 1996). The antrum increases in size, and granulosa cells differentiate into two sub-populations; the mural population and the cumulus population. Mural granulosa cells are large, steroid producing cells which line the outside of the follicle wall, compared to cumulus cells that are

small and non-steroid producing, and are in intimate contact with the oocyte (Amsterdam et al, 1975). The cumulus cells have been shown to be important regulators of maintenance of oocyte meiotic arrest. When oocytes are removed from the oocyte-cumulus complex prematurely oocytes undergo spontaneous resumption of meiosis (Pincus and Enzman, 1935). Oocytes taken from follicles at the preantral stage are incapable of resuming meiosis, but oocytes taken from antral follicles are capable of resuming meiosis (Sorensen and Wassarman, 1976; Wickramasinghe et al, 1991).

Follicle Development

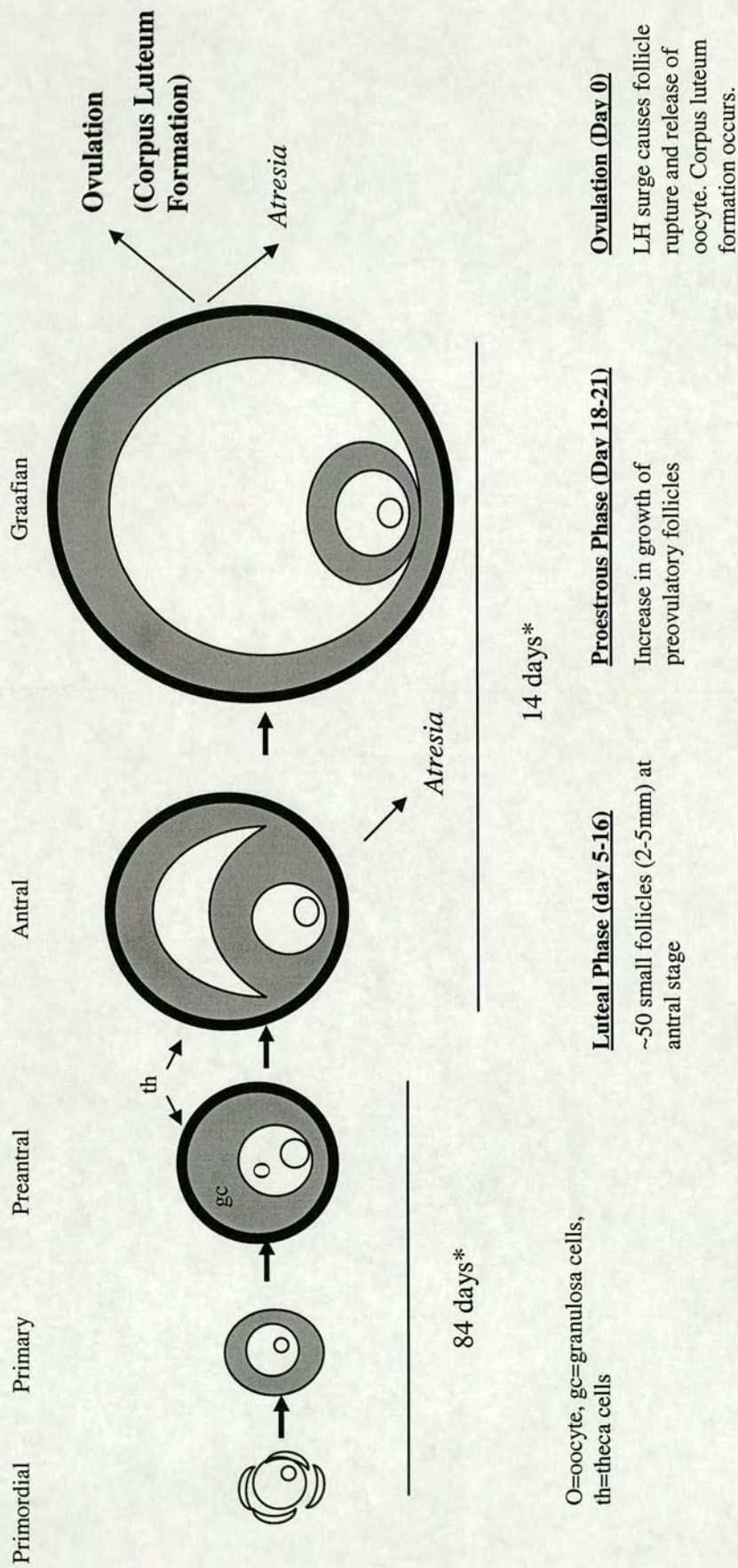


Figure 1.1: Follicle Development and the Oestrous Cycle
 (*Time-scale for follicle development taken from Morbeck et al, 1992)

1.5 Oocyte Development

Oocyte development takes place throughout folliculogenesis in a stage-dependent manner. Throughout folliculogenesis the oocyte must acquire the ability to resume meiosis in preparation for successful sperm penetration and fertilisation.

The oocyte matures throughout folliculogenesis, and in preparation for fertilisation must undergo complex cellular processes. During the transition from preantral to antral follicle, the oocyte acquires meiotic competence (Sorensen and Wassarman, 1976; Wickramasinghe et al, 1991). To ensure successful fertilisation the oocyte must complete the following processes:

meiosis; organelle production and re-distribution; RNA production; nuclear maturation and cytoplasmic growth and maturation (Hardy et al, 2000). If any of these processes are not completed successfully to an appropriate stage, fertilisation will not proceed correctly.

Oocyte development within the ovarian follicle is extremely important, and is a very lengthy process. Although preantral follicles contain oocytes which are developmentally incompetent, this stage of folliculogenesis is vital for oocyte development. During the preantral phase of follicle development oocytes synthesise molecules which are essential for successful resumption of meiosis such as cyclin B and mitogen-activated protein kinases (Chesnel and Eppig, 1995a, b; Christmann et al, 1994).

The majority of oocyte growth occurs before the development of the follicular antrum. At the primordial stage of development the oocyte is 30µm in diameter. By the time the follicle reaches the early antral stage of development the oocyte has almost reached its full size of 115µm. The successful development of the oocyte within the preantral follicle is therefore vitally important. Preantral

follicle development is a lengthy process, taking around 84 days in the pig (Morbeck et al, 1992). The oocyte is arrested at the dictyate stage of prophase I of meiosis during follicle development, and this is known as the germinal vesicle stage, when the oocyte has an intact nuclear membrane. At the time of ovulation, the luteinizing hormone (LH) surge triggers the release of the oocyte, by causing the follicle to rupture (see figure 1.2 for the LH surge and resumption of meiosis). In mammals when the LH surge is triggered *in vivo*, the germinal vesicle breaks down, and the oocyte is deemed to be a primary oocyte at the first meiotic division. Meiosis is resumed due to a decrease in intracellular cyclic adenosine monophosphate (cAMP) (Aktas et al, 1995; Eppig, 1996). Meiosis is subsequently suspended at metaphase II, and the oocyte is then ready for sperm penetration (Moor and Dai, 2001).

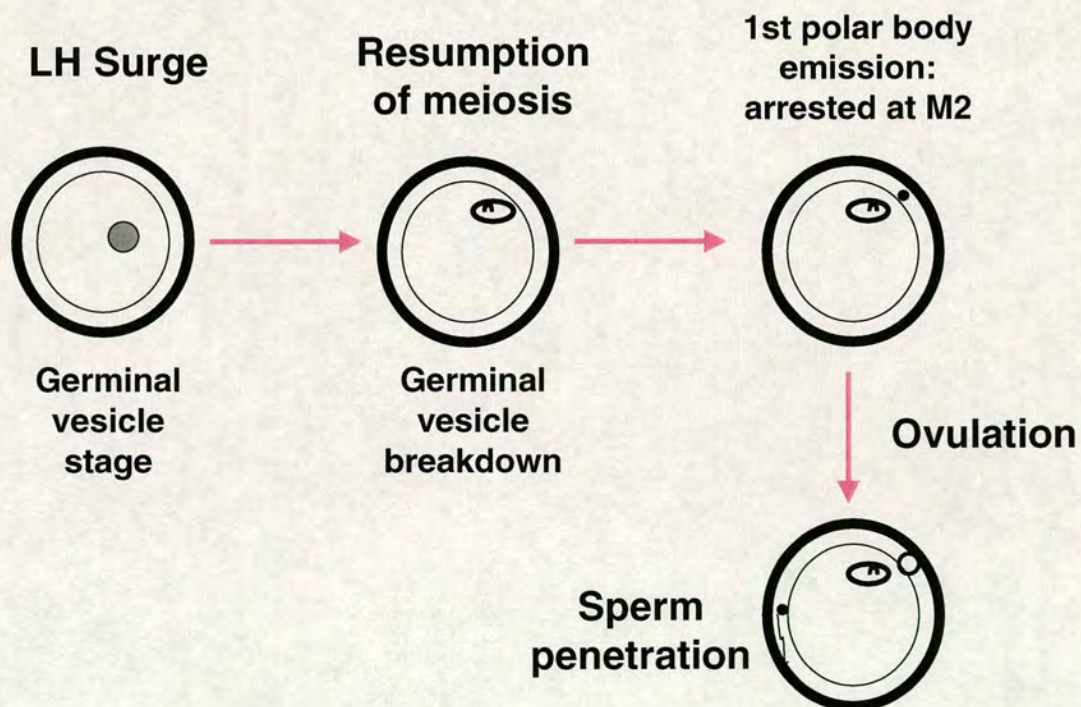


Figure 1.2: Oocyte Meiosis Resumption: Resumption of meiosis in oocytes at the LH surge.

Ovulation itself is dynamic event which requires the bursting of healthy tissue. For many years it was presumed that the follicle unit burst due to an increase in pressure, however, for the past two decades it has largely been considered to be an inflammatory response by ovulatory follicles, which occurs after pituitary gonadotrophin stimulation (Espey, 1980). Many of the changes which occur in the ovulatory follicle, such as vascular changes and increased blood flow to the area, are associated with the inflammatory response. There is also oedema and increased prostaglandin production at the area of ovulation, indicating that ovulation is indeed an inflammatory event (Espey et al, 1981).

Oocyte size is a vital factor in determining whether or not an oocyte is capable of resuming meiosis. As the follicle size increases there is a direct correlation between follicle size and the percentage of oocytes successfully resuming meiosis (Tsafiriri & Channing, 1975; Motlik et al, 1984). This is important in the consideration of culturing follicles *in vitro*, as preantral follicles contain oocytes that are developmentally incompetent at the start of culture. Developmental competence of the oocyte can be judged by its chromosome configuration.

Porcine oocytes were first matured *in vitro* by Edwards (1965). Breakdown of the germinal vesicle, which is the first sign of the resumption of meiosis, was initially reported by Motlik and Fulka (1976). Motlik and Fulka conducted experiments to ascertain the changes that occurred in the oocyte during germinal vesicle breakdown and up to metaphase II. This was a means of assessing whether or not oocytes had resumed meiosis, and a useful tool for quantifying the resumption of meiosis *in vitro*. Hirao et al (1994) showed for the first time that porcine oocytes taken from follicles removed from the ovary before reaching the antral stage could resume meiosis. Follicles were removed from the ovary

and cultured over periods of up to sixteen days *in vitro*. The diameter of over half of the oocytes after this period was $>100\mu\text{m}$. It was found that oocytes of less than $90\mu\text{m}$ were highly unlikely to resume meiosis and that the closer the oocyte was to full size, the greater the chance of the oocyte gaining competence. It has been shown that more than 95% of oocytes $<90\mu\text{m}$ are incapable of resuming meiosis (Hirao et al, 1994).

1.6 Oocyte-Follicle Interactions

It has long been known that the somatic cells of the follicle regulate oocyte development, and deficient contact between the oocyte and somatic cells results in the death of the oocyte (see figure 1.3 for follicle-oocyte interactions, adapted from Eppig, 2001). The somatic cells of the follicle hold the oocyte in meiotic arrest, and early removal of the oocyte from the follicular unit results in the premature resumption of meiosis (Pincus and Enzman, 1935; Racowsky and Baldwin, 1989). The somatic cells of the follicle are also important to provide nutrients for the oocyte, and the required influx of metabolites (Eppig, 1991). The oocyte and follicle are attached through gap junctional contacts, and disruption of this contact results in death of the oocyte. Connexin43 is the most common gap junctional contact between cumulus cells and the oocyte, and follicle development is arrested at the preantral stage in mice lacking in this gene, and oocytes are developmentally incompetent (Ackert et al, 2001).

For many years the oocyte was considered to be a passenger within the follicle, and was thought to have no effect on somatic cell development. This idea has been disproved, as the oocyte has been shown to exert control over the follicle thereby controlling its own environment (Eppig et al, 2002; Matzuk et al, 2002). The oocyte has been shown to secrete factors controlling such processes as cumulus cell expansion (Buccione et al, 1990; Vanderhyden et al 1990; Vanderhyden, 1993); proliferation of preantral follicles (Vanderhyden et al, 1992); and to control gene expression in surrounding cumulus cells (Eppig et al, 1997).

A body of work to assess the potential role of the oocyte in follicle development was carried out in the early 1990's. Experiments were carried out which involved

the culture of murine oocyctomized complexes (oocyte-cumulus complexes which have had the oocyte microsurgically removed, OOX) with FSH to assess whether the oocyte had any effect on cumulus expansion. Initial experiments revealed that mouse OOX could not undergo cumulus expansion in the absence of the oocyte (Buccione et al, 1990; Salustri et al, 1990; Vanderhyden et al, 1990). It was concluded from these experiments that a factor was produced by the oocytes that allowed cumulus cell expansion to occur in the presence of FSH. It was also concluded that this factor was diffusible, as the OOX could undergo cumulus expansion when cultured in medium conditioned with denuded oocytes. This experiment was repeated to look at the role of the oocyte in porcine cumulus cell expansion. It was found that oocyctomy has no effect on the expansion of cumulus cells in the pig, in contrast to similar experiments in mice (Prochazka et al, 1991; Nagyova et al, 1999).

In mice it has therefore been established that an oocyte-secreted factor is responsible for this process, in response to FSH. Similar experiments done in the pig however, revealed that this process is independent of oocyte-secreted factors. The synthesis of hyaluronic acid (HA) in the porcine is dependent on FSH, but oocyctomy does not have an effect on its production (Prochazka et al, 1991; Nagyova et al, 1999). It was concluded however, that a cumulus expansion enabling factor is produced by porcine oocytes, as mouse OOX can still undergo cumulus cell expansion in medium conditioned with porcine oocytes. In the bovine it has also been found that the oocyte produces a cumulus-expansion enabling factor (Ralph et al, 1995). A similar effect to the porcine was found on cumulus expansion in the cow. Bovine cumulus cells can expand independently of the oocyte, but bovine oocytes secrete the cumulus expansion enabling factor

required by murine cumulus cells, and this factor is not inactivated by the effects of freezing (Ralph et al, 1995).

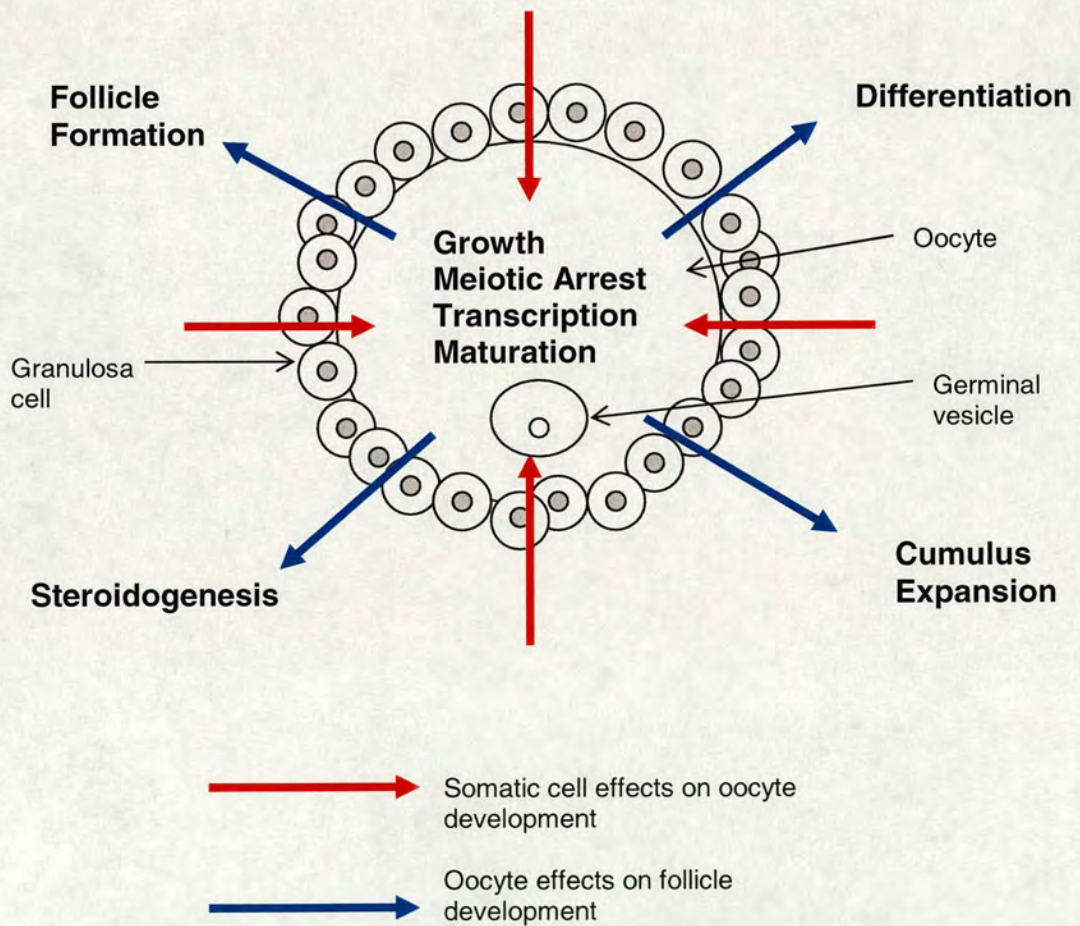
Whilst a function for oocyte secreted factors had been found, the identity of these factors was not (Eppig et al, 1993). It was the use of molecular techniques that identified Growth and Differentiation Factor-9 (GDF-9) as an oocyte-specific factor (McGrath et al, 1995), and that led to the hypothesis that GDF-9 was involved in regulating somatic cell function. Studies using GDF-9 knock-out mice showed that mice deficient for this gene are infertile due to a block in folliculogenesis at the primary stage (Dong et al, 1996). GDF-9 β is a close relation of GDF-9, and a member of the Transforming growth factor- β (TGF- β) superfamily of growth factors, and is also known as bone morphogenetic protein-15 (BMP-15). It has been shown to be expressed exclusively in the oocyte in the mouse and human (Dube et al, 1998). A natural mutation of the BMP-15 gene exists in sheep, and homozygotes of the mutation display impaired follicle production past the primary stage (Galloway et al, 2000). In contrast heterozygotes for the condition have an increased ovulation rate, and it has been postulated that mutants with an inconsistency in the receptor gene display increased ovulation rates due to a possible increase in SMAD phosphorylation (Souza et al, 2001). Smads are mammalian homologues of *Drosophila* Mad and *C. elegans* Sma. The Smad proteins are transcription factors which act as TGF- β receptor substrates (Heldin et al, 1997). Smads, for example Smad 2, are anchored to the cell membrane before the receptor is activated. When the receptor is activated by a hormone or growth factor it phosphorylates the Smad, which then dissociates from the cell membrane. The phosphorylated Smad will then form a heterocomplex with another Smad, e.g. Smad 2 with Smad 4. This

complex will then translocate to the nucleus, and bind to the promoter region of target genes, thus regulating gene expression (Chen et al, 2002).

Another knock-out model which has been used to look at the effect of the oocyte on fertility is Factor in the Germline alpha (FIG- α). FIG- α is a helix-loop-helix transcription factor that has been studied using knock-out mouse models. When this gene is knocked out, primordial follicle formation does not take place.

Oocyte stocks are therefore depleted and this results in female infertility (Soyal et al, 2000).

Figure 1.3: Oocyte-Follicle Interactions: Follicle and oocyte interactions, based on Eppig (2001).



1.7 Hormones and Growth Factors in Folliculogenesis

Follicular growth and development is regulated by a combination of endocrine and paracrine factors. Hormones and growth factors are produced either locally or by the endocrine glands, and are regulated through positive and negative feedback mechanisms. The four major systems or organs responsible for the production of the reproductive hormones involved in female reproduction are the hypothalamus, the pituitary gland, the ovary and the uterus. The glycoprotein family of hormones are vital in the regulation of various reproductive processes. The glycoproteins consist of the pituitary hormones; follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and the placental hormone chorionic gonadotrophin (CG), which exists only in humans and equids. These hormones are very similar in structure, and each contains a common α subunit, which is non-covalently associated with a hormone specific β subunit (Cupps, 1991).

1.7.1 Pituitary Hormones: FSH and LH

FSH is one of the principle regulators of folliculogenesis and ovulation (Hillier, 2001). FSH works in concert with LH to stimulate the ovary to produce oestrogen (Armstrong et al, 1979). It is produced by the anterior pituitary, and stimulates the growth of antral follicles. The hormone is a heterodimeric glycoprotein that consists of a common α sub unit, and a hormone-specific β subunit. The common α subunit contains 10 cysteines, and these are involved in intrasubunit disulfide linkages and two N-linked glycosylation sites. (Themmen and Huhtaniemi, 2000). Although the β subunit is specific to each particular hormone, there is a large amount of homology among the different glycoproteins.

The β sub unit is smaller than the α subunit, and all possess three exons (Cupps, 1991).

FSH is known to be essential for follicle development beyond the preantral stage. This has been shown using a mouse knock-out model for the FSH receptor (FSH-R) gene. Mice which are null for the FSH-R gene are infertile due to a block in folliculogenesis at the preantral stage (Kumar et al, 1997), and they also display abnormal gene expression. In the mouse FSH has been shown to be essential for the development of an antral cavity *in vitro*, indicating FSH probably plays an important role in follicle differentiation (Gore-Langton and Daniel, 1990; Cortvrindt et al, 1997). *In vivo* it has been shown in sheep that removal of the pituitary gland results in widespread atresia of antral follicles after just four days due to a lack of FSH (Dufour et al, 1979). In the human it has recently been discovered that FSH receptors are present in the oocyte (Meduri et al, 2002). For many years it was thought that any actions of FSH on the oocyte came secondarily through the granulosa cells, but it now appears that this effect may be directly exerted upon the oocyte. It appears therefore, from both *in vitro* and *in vivo* studies, that follicle development is profoundly affected by FSH.

LH is also produced by the anterior pituitary and consists of the common α -sub-unit, and a hormone-specific β sub-unit. As mentioned above, LH and FSH work synergistically to produce oestrogen. LH stimulates the theca cells to produce androgen, which then acts as a substrate for oestrogen production by the granulosa cells. This is known as the two cell-two gonadotrophin mechanism of steroid production (Armstrong et al, 1979). LH receptors are present in the theca cells of the follicle, and also in the granulosa cells of follicles approaching the

ovulatory stage of development (Peng et al, 1991; Bao et al, 1997; Manikkam et al, 2001).

The mechanism of action of the 2-cell 2-gonadotrophin theory is displayed in figure 1.4. The two-cell nature of steroid production was identified when it was discovered that isolated porcine granulosa cells converted pregnenolone to progestins, but not to androgens. However, the granulosa cells could convert exogenous testosterone and androstenedione through aromatisation with P450 aromatase in response to FSH. It was also found that 17β -hydroxysteroid dehydrogenase (17β HSD) converts progestins to androgens in a specific action in the theca cells by LH stimulation. It was then proposed that the steroid precursors are produced in the theca and transferred to the granulosa cells to be converted to testosterone (Gore-Langton and Armstrong, 1994).

The 2-cell 2-gonadotrophin model of oestrogen synthesis involves the conversion of cholesterol into oestradiol through LH stimulation of the theca cells and FSH stimulation of the granulosa cells. The cell cooperation which is required to convert cholesterol to oestrogen requires that androgens which are synthesised by the theca cells in response to LH diffuse across the basement membrane which separates the different cell compartments. This androgen is then aromatised into oestrogen by FSH stimulation of the granulosa cells (Gore-Langton and Armstrong, 1994).

The conversion of cholesterol to oestradiol is not a one-step process, and there are several intermediary steroids which are produced, and the separate steps of steroid synthesis are catalysed by different enzymes. The rate limiting step in the conversion of cholesterol to steroids is thought to occur when cholesterol is converted to pregnenolone by P450 cholesterol side-chain cleavage (P450

CSCC; figure 1.4). The source of cholesterol for steroid synthesis comes from several sources. The main source, however, comes from preformed cholesterol in the blood, which is mainly present in the form of circulating lipoproteins (Strauss et al, 1981). In all mammals, the principal cell types involved in the synthesis of sex steroids are the granulosa and theca cells, as mentioned above. The cells which respond to LH stimulation are the theca cells, and the interstitial cells of the ovarian stroma. The FSH responsive cells are the mural granulosa cells. LH stimulation of theca cells results in an increase in the production of cAMP. This acts as the main stimulus for these steroid activities. FSH-induced aromatisation of androstenedione regulates aromatase enzyme activity, resulting in the synthesis of oestrogen (Gore-Langton and Armstrong, 1994).

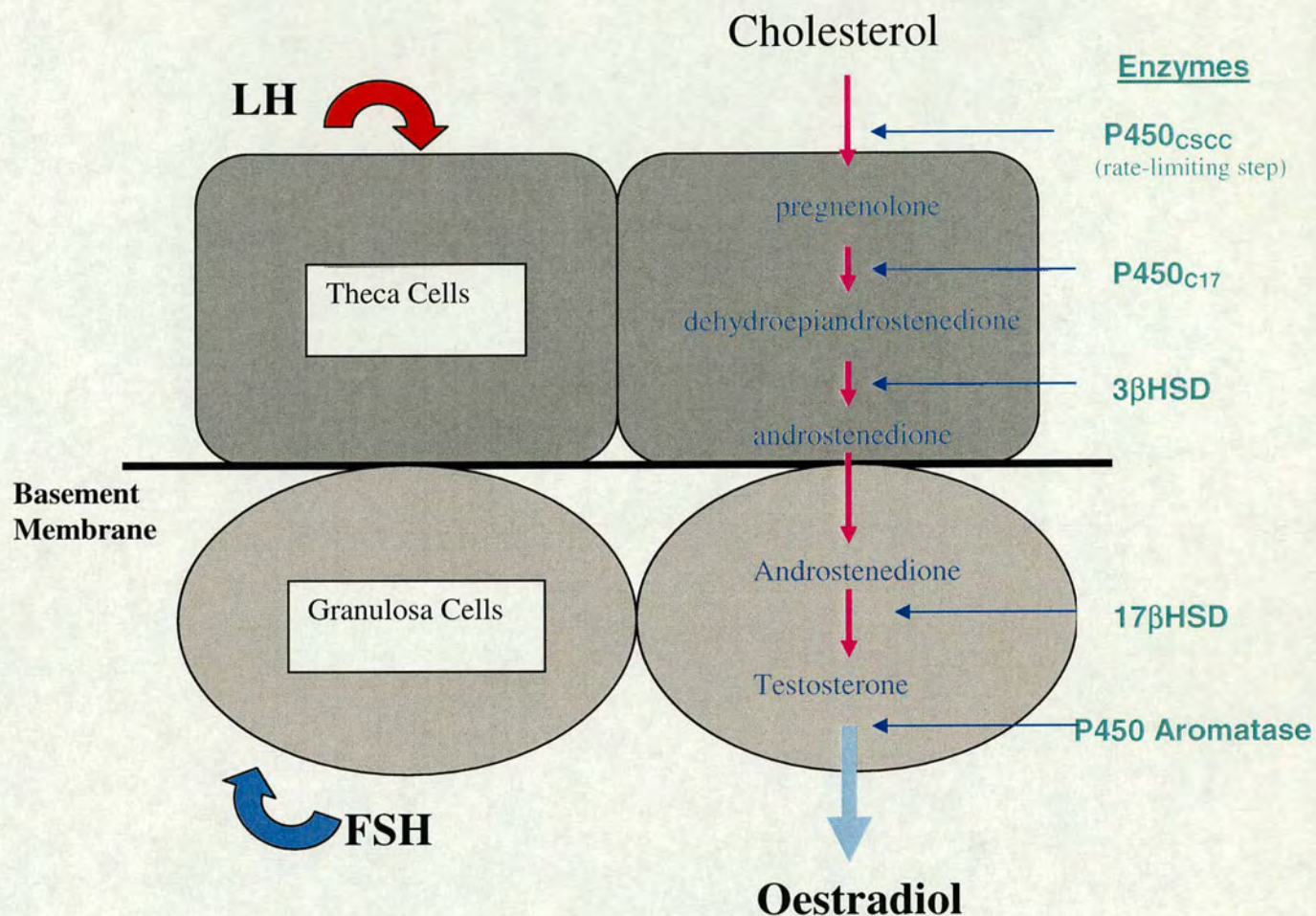


Figure 1.4: 2-Cell 2-Gonadotrophin Mechanism The 2-cell 2-gonadotrophin theory of oestrogen production, showing the production of oestradiol through androgen production in the theca cells, which is transported to the granulosa cells and aromatase converts it to oestradiol (adapted from Fowler, 2003, with additions from Berne and Levy, 1996).

(P450 CSCC= P450 cholesterol side chain cleavage enzyme;

P450C17=17αhydroxylase; 3βHSD= hydroxysteroid dehydrogenase; 17βHSD= 17β hydroxysteroid dehydrogenase.)

1.7.2 Inhibin and Activin

Activins and inhibins are members of the TGF- β family, and have opposing roles within the ovary in the control of FSH secretion by the pituitary gland (Findlay, 1993). Activin stimulates the release of FSH from the pituitary and inhibin suppresses it (Rivier et al, 1986; Ling et al, 1986). Inhibin was first described about seventy years ago when it was discovered that when rat seminiferous tubules were destroyed, the pituitary hypertrophied (McCullagh, 1932). Studies showed that a factor was being over-produced, and this was later found to be FSH. It was concluded that inhibin was produced to control the release of FSH in the gonads (Channing et al, 1985). Activin and follistatin were subsequently identified, and activin was found to increase FSH production, and follistatin bound activin, and rendered it inactive (Vale et al, 1988; Woodruff and Mather, 1995).

Inhibin and activin are dimeric in structure, and share a homologous β -subunit. Three related protein subunits, known as α , β_A and β_B , make up the activins and inhibins, through different combinations of the three related subunits. Two forms of inhibin exist, A and B, and these are formed by heterodimerization of the α subunit and either the β_A or β_B subunit. Three forms of activin exist, and these are formed by dimerization of the β subunits. (Ling et al, 1986; Mason et al, 1986; Mayo et al, 1986; Vale et al, 1986). Follistatin is a monomeric glycoprotein that forms an almost irreversible inactive complex with activin, and neutralises its actions (Sidis et al, 2002).

1.7.3 IGF System

The insulin-like growth factor system (IGF system) is a complex family of growth factors which interact with binding proteins to control steroidogenesis and somatic cell differentiation (Giudice, 2001; Huang et al, 2001; Wright et al 2002). The IGFs interact with specific receptors and IGF- binding proteins control their bioavailability. It has been shown in various species that the IGFs stimulate granulosa cell proliferation and steroidogenesis (Monget et al, 2002), but *in vitro* they have been shown to have a detrimental effect on oocyte size in bovine preantral cultured follicles (Thomas et al, 2001). Recent research has shown that there are important functional links between IGF-1, oestradiol, and FSH signalling (Sun et al, 2003). It has been found that IGF-1, IGF-1 receptor, and FSHR co-localize to the granulosa cells of small growing follicles and preovulatory follicles (Zhou et al, 1997). Growth factors and hormones have also been implicated in the regulation of cell death in the ovary. There is a constant turnover in the ovary, which sees more than 99% of follicles die through apoptosis. This apoptosis is thought to be controlled in part through the actions of gonadotrophins and growth factors.

1.7.4 EGF

Epidermal growth factor (EGF) is a growth factor which has been implicated in early follicle development. In the cow it has been found to be beneficial to survival of preantral follicles *in vitro* (Gutierrez et al, 2000), and it has been found to enhance the expression of Connexin 43 (Cx43, an important gap junctional contact between the oocyte and the somatic cells) in porcine follicles (Bolamba et al, 2002).

1.8 Follicular Cell Death

1.8.1 Apoptosis

Programmed cell death is an essential regulatory mechanism that controls homeostasis of adult tissue, and is responsible for the removal of infected, transformed or damaged cells. Apoptosis is one form of programmed cell death, and is characterised by maintenance of intact cell membranes, to allow surrounding cells to engulf the dying cell to avoid the production of a local inflammatory response.

1.8.2 Cell Death in Ovarian Follicles

As has been mentioned previously, the number of germ cells present before birth rises rapidly in the foetal pig, and then declines sharply to leave a resting population of primordial follicles of around 500,000 (Erickson, 1967; Black and Erickson, 1968). The loss of primordial follicles which can be observed before birth is due to widespread atresia. The remaining primordial follicles represent the entire genetic store for the mammalian female at birth, however less than 1% of these oocytes will ever be ovulated due to atresia. Follicles are recruited to grow throughout the reproductive life span of the female, through an unknown signal. Once follicles are recruited into the growth pool they will either reach the preovulatory stage and release the oocyte for fertilisation, or degenerate and die. The latter is by far the most likely fate for developing follicles. Follicular atresia is thought to occur through an apoptotic mechanism.

The prevalent form of cell death in the ovarian follicle is granulosa cell apoptosis (Hughes and Gorospe, 1991; Tilly et al, 1991; Guthrie et al, 1995). Follicular

atresia has been found to be lowest in primordial follicle populations ($\leq 6\%$) and preantral follicles ($\leq 17\%$), and highest in antral follicles, at around 55% (Erickson, 1967; Dalin, 1987). Similar results were found in the granulosa cells of pig follicles by Telfer and Ghafari (unpublished) where it was found that follicles in the primordial and preantral stages of development displayed little cell death, but follicles which were larger than $500\mu\text{m}$ displayed $>50\%$ apoptosis.

Apoptosis is mediated by two families of proteins; the caspases and the Bcl/Bax family. The caspases function as proteases, and there are more than 100 known substrates for this family of apoptotic mediators, including laminins and cytoskeletal proteins. One of the main functions of the caspases is to activate caspase-activated DNase (CAD), which causes DNA fragmentation, one of the hallmarks of apoptosis (Markstrom et al, 2002). The Bcl-2 family of apoptotic mediators have both anti-apoptotic and pro-apoptotic members. The anti-apoptotic factors include Bcl-2 and Bcl-X_L. Pro-apoptotic members include Bax, Bid, Bik, BOD and Bcl-X_S. Many members of the Bcl-2 family have been isolated in the ovary (Hsu and Hsueh, 2000), and their function appears to be to control the release of pro-apoptotic factors, such as cytochrome c, from the mitochondrion into the cytosol (Antonsson and Martinou, 2000).

There are several factors that have been identified as apoptosis inducers or suppressers. Apoptosis inducers include Tumour necrosis factor- α (TNF α) (Sasson et al, 2002) and Gonadotrophin releasing hormone (GnRH) (Andreu et al, 1998; Parborell et al, 2001). Suppressers of apoptosis include LH, FSH, Insulin-like Growth Factor-1 (IGF-1), Epidermal Growth Factor (EGF), Transforming Growth Factor- α (TGF- α), bFGF, keratinocyte growth factor,

interleukin 1 β , and ascorbate (Tilly et al, 1992b; Chun et al, 1994; Tilly et al, 1995; McGee et al, 1999; Thomas et al, 2001; Murray et al, 2001). Ascorbate is of particular interest as an anti-apoptotic factor. It has been shown to reduce cell death in populations of somatic cells in rodents and cows in *vitro* (Thomas et al, 2001; Murray et al, 2001).

1.9 Ascorbate

Ascorbic acid (vitamin C) is a very unstable vitamin that readily undergoes oxidation. It is a ketolactone which ionises in two stages. The first pKa value is ~4.2 at 37°C, and the second is ~11.6 (Levine and Morita, 1985). At physiological pH therefore, ascorbate is almost completely in its anionic form. It is thought that ascorbate functions as an anti-oxidant in biological systems by donating electrons while undergoing reversible oxidation to dehydroascorbic acid (Bielski et al, 1975).

Ascorbate has long been linked with fertility (Kramer et al, 1933; Deane, 1952), and is a dietary requirement for several mammals, although it is synthesised naturally in the pig (Grollman and Lehninger, 1957). Species incapable of synthesizing ascorbate lack an essential enzyme which catalyses the conversion of L-gulono- γ -lactone to ascorbate. Ascorbate is known to play an essential role in female reproduction as guinea pigs with scurvy (a severe lack of vitamin C), display severe follicle degeneration and are anovulatory (Kramer et al, 1933). The ovaries are a site for ascorbate accumulation. Studies have shown that ascorbate build up in the ovaries occurs in granulosa, theca, and luteal cells of the corpus luteum, and also in the oocyte (Hoch-Ligeti and Bourne, 1948; Deane, 1952).

Ascorbate has been shown to have three main roles in the ovary; collagen synthesis, steroidal, and as an anti-oxidant. Initial studies were carried out on ascorbate looking at its role in the synthesis of collagen, and it was found that ascorbate was necessary for tissue development and also at sites of damaged tissue. Through an action as an electron donor, ascorbate is a co-factor for

processing of pro-collagen (Pinnell, 1985). It has been suggested that ascorbate is also involved in the promotion of collagen production at the level of the gene (Padh et al, 1991).

A putative role for ascorbate in the regulation of steroidogenesis has been suggested (Sanyal & Datta, 1979). Experiments showed that ascorbate could inhibit the production of steroids. This was not found in later experiments on the cow and mouse (Thomas et al, 2001; Murray et al, 2001). Addition of ascorbate to serum-free medium in cultured cow and mouse preantral follicles had no effect on the production of oestradiol in comparison to control groups. Ascorbate content in the ovaries has been shown to be controlled by LH concentration. LH blocks the uptake of ascorbate by gonadotrophin-primed rat ovaries (Stansfield and Flint, 1967). Use of steroidal contraceptives in women lowers the plasma concentration of ascorbate (Rivers and Devine, 1975) so there is clearly steroidogenic control over ascorbate concentration.

A possible explanation for the high ovarian content of ascorbate is the tremendous amount of tissue remodelling required for folliculogenesis. Tissue remodelling produces large amounts of free radicals, and has a high requirement for collagen synthesis. As ascorbate has been shown to be involved in the mopping up of free radicals, and is required for the synthesis of collagen, which is another important process in folliculogenesis, it seems likely that it would be involved.

1.10 Tissue Remodelling in the Ovary and the MMP

System

The ovary is a dynamic structure, which is equipped to deal with constant turnover and change of structure. Constant remodelling of the basement membrane is required to deal with cyclical changes that cause follicular development to take place. Growth of the ovarian follicle requires profound cellular proliferation, and structural re-modelling in the basement membrane. A class of proteolytic enzymes known as the matrix metalloproteinases (MMPs) maintain homeostasis of the basement membrane. MMPs are a class of enzymes that are responsible for the breakdown of the proteinaceous compounds of the membrane. They were first discovered when it was found that tadpole tail fragments produced a diffusible enzyme that was capable of degrading a collagen gel (Gross and Lapiere, 1962). The activity of MMPs is regulated by their endogenous inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs) (Curry and Osteen, 2001; Sternlicht and Werb, 2001).

The MMPs are a group of related proteins that depend on zinc to degrade the extracellular matrix (ECM). They are secreted into the ECM as proenzymes, and are subsequently activated by proteolytic cleavage. The matrix metalloproteinase enzymes are a precisely regulated group of enzymes, and together with their endogenous inhibitors, TIMPs, they regulate many diverse processes in the body, including embryogenesis (Zhang et al 2003), wound repair (Klein et al, 2002) and cancer (Edwards et al, 2003). The MMP system is associated with processes where there are large amounts of tissue remodelling required.

There are four types of MMPs; the gelatinases, stromelysins, membrane-type, and the collagenases (Nagase and Woessner, 1999). The MMPs which have been

associated with follicle development are the gelatinases (MMP-2 and MMP-9), as they are able to potently cleave the protein components of the basement membrane (Sternlicht and Werb, 2001). A major component of the follicle basement membrane is collagen IV. As MMP-2 and MMP-9 can potently bind and cleave collagen IV it seems likely they are involved in follicle development. The structure of the gelatinases is shown in figure 1.5 (adapted from Sternlicht and Werb, 2001). These proteolytic enzymes consist of a pre domain, which directs synthesis to the endoplasmic reticulum, a pro domain which maintains the enzymes latency, and the catalytic domain which contains the zinc binding site. There is also a hemopexin domain which is coupled to the catalytic domain by a hinge region. The hemopexin domain is involved in substrate and TIMP binding. The gelatinases are different from other MMPs as they contain three fibronectin-type inserts which potently bind to collagen. These inserts are found within the catalytic domain.

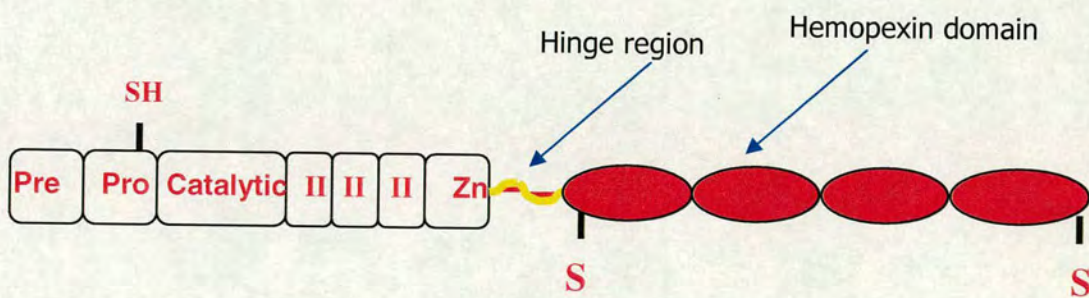


Figure 1.5: Structure of the Gelatinase MMPs. (Adapted from Sternlicht and Werb, 2001).

The activity of the MMPs is controlled by two groups of enzymes; serum-borne, and tissue derived inhibitors. (Gomez et al, 1997). Serum-borne inhibitors of metalloproteinase action include α -2 macroglobulin, and these molecules act to inhibit MMPs in the fluid phase. TIMPs are tissue inhibitors of metalloproteinases. TIMPs are two-domain molecules which inhibit MMP action by forming a stable native molecule (Huang et al, 1996). Four TIMPs have been identified to date; 1, 2, 3, and 4. TIMP-1 has been found in the rat oocyte (Bagavandoss, 1998).

Ovarian follicular development requires extensive remodelling of the extracellular matrix, especially at the latter stages of follicle development, when dynamic changes occur very quickly. The role of the MMP system in follicles at the peri-ovulatory stage of development is well-documented. The general consensus is that gelatinase activity increases significantly towards ovulation, for example in the rat there is 4-5 fold increase in gelatinase activity in follicles after LH stimulation (Curry et al, 1992). In the human a similar increase in gelatinase activity can be observed close to ovulation (Puistola et al, 1986), and a similar increase in MMP-2 is seen in ovine follicular fluid (Gottsch et al, 2002). MMP and TIMP expression patterns in the rat suggest a role in follicle wall degradation at ovulation (Hagglund et al, 1999). Bagavandoss (1998) noted an up-regulation of expression of MMP-2 and MMP-9 as follicle development progresses.

The evidence points to a significant role for the MMPs in ovulation, but a role in early folliculogenesis is less well documented. A putative role for the MMP system has been suggested for early follicle development, but this has yet to be proven. Most studies have concentrated on follicle development after the development of the antrum when studying folliculogenesis and the MMP system.

However, in the cow it has been found that MMP-2 and MMP-9 are secreted by cultured bovine preantral follicles. Furthermore, in this study McCaffery et al (2000) found that when MMP-9, TIMP-1 and TIMP-2 were produced by cultured bovine preantral follicles there was a greater chance of follicle somatic cell health, highlighting a potential role for MMPs and TIMPs as secreted markers of follicle health.

1.11 Follicle Culture Systems

1.11.1 Why Culture Preantral Follicles?

The primordial follicles present in the ovary after birth represent the entire genetic store in the female mammal; however less than 1% of these follicles will ever be ovulated. This represents a tremendous waste of genetic material *in vivo*, especially of genetically valuable domestic animals, or in women who cannot conceive naturally. Ideally the vast numbers of primordial follicles which are present within the ovary could be harvested and grown *in vitro*, as these are the most homogeneous starting population of follicles, and are present in large numbers (100's of 1000's). It is not known if the vast amount of cell death which is observed *in vivo* is inherent in certain follicles which will be destined to die, or whether it is an environmental effect rather than genetic programming which causes the widespread atresia which can be observed. This atresia which is observed in the ovary is far more prevalent in follicles at the antral stage of development. Levels of cell death in primordial and preantral follicles are far lower than in follicles at a later stage of development (Guthrie and Garratt, 2001).

Problems are associated with the culture of primordial follicles, as they are relatively hormone insensitive and difficult to culture, unless done so in strips of ovarian cortical tissue or whole organ culture (cortical strips: Wandji et al, 1996; Braw-Tal and Yossefi, 1997; Hovatta et al, 1997; Wandji et al, 1997; Whole ovary culture: Eppig and O'Brien, 1996; O'Brien et al, 2003). The next stage of follicle development to be considered for culture is the preantral range. Follicles at this stage of development still represent a suitable starting population for use

in culture experiments as they are homogeneous and display low levels of cell death.

Although the use of early follicles to provide large numbers of quality germ cells is a long term aim of *in vitro* culture groups, it is irrelevant for the purposes of this thesis, as such a culture system is far from becoming a reality at the present time. Rather, for the purpose of this thesis, *in vitro* culture systems can be used to look at the effects of specific factors on early folliculogenesis, in order to gain a clearer picture of the events involved in these processes.

1.11.2 Methods for the Isolation and Culture of Ovarian Follicles

Methods for *in vitro* follicle culture:

- (1) Primordial follicle culture through either whole ovary culture or strip culture.
- (2) Granulosa-oocyte complex culture by dissecting out preantral follicles from enzyme-treated ovarian tissue.
- (3) Whole follicle culture using intact preantral follicles dissected out from ovarian tissue without the use of enzymatic degradation.

1.11.3 Whole Ovary and Strip Culture

The culture of whole ovaries in the mouse has led to the successful production of live offspring (Eppig and O'Brien, 1996; O'Brien et al, 2003). This method is not applicable for larger animals however, as large organs would make gas exchange impossible, and the long length of time for follicular development for the domestic species in comparison to rodents make this seem impossible. There is a relatively high success rate using this procedure in the mouse, however.

Initial experiments using this system produced only one live mouse (Eppig and

O'Brien, 1996), in a system which cultured whole ovaries for 8 days. Ovaries were then disaggregated using collagenase and the granulosa-oocyte complexes were cultured for a further 14 days. The one resulting live mouse from these cultures displayed severe abnormalities. A revised protocol for this technique however, has recently resulted in the birth of 59 live young (O'Brien et al, 2003). Ovarian strip culture is a useful tool to investigate the recruitment of primordial follicles into the growing pool. It provides an alternative method to whole ovary culture in large mammals, and has been used to study initiation of follicle growth in cattle (Wandji et al, 1996; Braw-Tal and Yossefi, 1997) baboons (Wandji et al, 1997), and human (Hovatta et al, 1997). Most primordial follicles activate in these culture systems, so they provide a useful tool for identifying inhibitors of follicle initiation.

1.11.4 Preantral Follicle Culture

There are two main methods which can be used for the isolation of preantral follicles and are applicable to the domestic species; enzymatic isolation using a collagenase tissue digestion, and micro-dissection using fine needles. These methods of culture are displayed in figure 1.6. Using these techniques follicles are isolated from thin ovarian strips. Enzymatic digestion causes breakdown of the basement membrane and allows the collection of granulosa-oocyte complexes. Rabbit follicles initially isolated in this manner were found to be normal (Nicosia et al, 1975). The technique was then transferred for use in rodents. It was found that large numbers of preantral mouse follicles could be isolated using a combination of mild collagenase and mechanical dissection

(Eppig & Schroeder, 1989; Torrance et al, 1989). This technique allows the collection of large numbers of early stage preantral follicles.

Isolation of follicles from the ovaries of larger mammals is more difficult. Thick stromal tissue renders techniques which are successful in rodents useless in other species. Oocyte quality is not found to be compromised in rodents using enzymatic dissection; however this has been found not to be the case in the domestic species. Collagenase treatment disrupts the basement membrane and the structural integrity of the follicle, and is thought to adversely affect oocyte viability (Telfer, 1996). The most reliable method is to isolate whole follicles using micro dissection of ovarian cortical strips, which although time-consuming with a relatively low yield, does not compromise follicular integrity or oocyte quality. Mechanical techniques have been found most useful in the isolation of follicles in the domestic species (McCaffery et al, 2000; Thomas et al, 2001).

Culture Systems

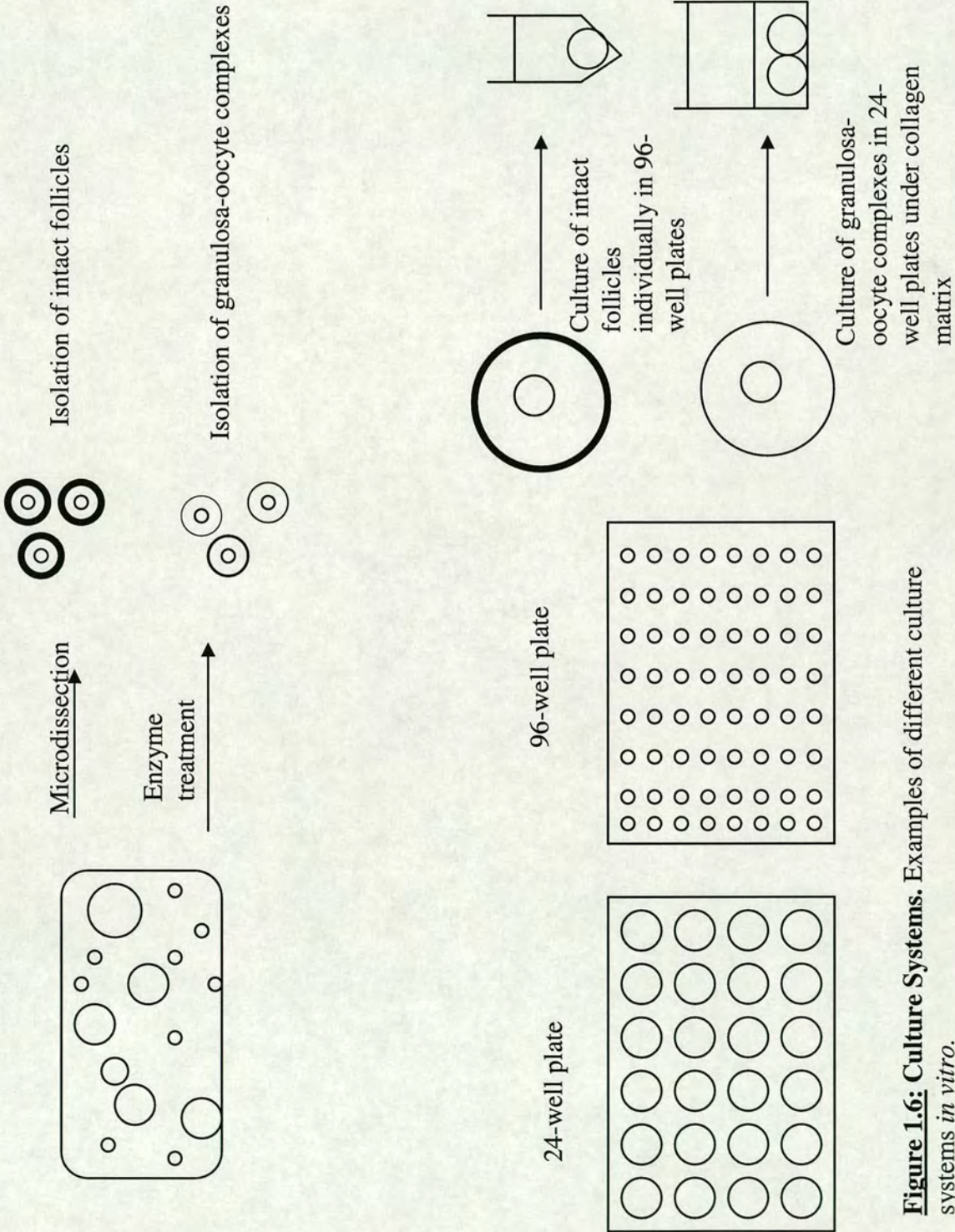


Figure 1.6: Culture Systems. Examples of different culture systems *in vitro*.

1.11.5 Rodent Systems

The culture of early follicles has been studied most extensively using rodent systems, with by far the most success. Rodents provide a useful model to study follicle development for several reasons. The short period required for the development of follicles in rodents from the primordial right through to producing fertilisable eggs means that a culture system has to support growth and development for weeks rather than months, which is the timescale for follicle development in the domestic species. Follicles are also easier to obtain in these species due to less dense stromal tissue, and follicles can be obtained in large numbers using enzymatic degradation. Follicles are also smaller, growing to about $600\mu\text{m}$, in comparison to preovulatory follicles in the domestic species, which can grow up to a few mm in diameter, which makes diffusion and gas exchange much less efficient than in the mouse.

Early experiments on hamster ovarian follicular development *in vitro* by Roy and Greenwald (1985) showed that hamster follicles could be isolated when minced ovaries were incubated in the presence of collagenase. These experiments were followed up by taking the isolated follicles and culturing them *in vitro* (Roy and Greenwald, 1989). Using a serum-free system it was found that the addition of gonadotrophins enhanced follicle development. Follicles from mice ovaries have been used in similar experiments, where mouse oocyte-granulosa cell complexes from preantral follicles were cultured *in vitro* by Eppig and Schroeder (1989). This system produced live young. Subsequent experiments cultured whole new-born mouse ovaries. New-born mice have only primordial follicles in their ovaries. Live young were produced after the culture of primordial follicles in whole ovary culture (Eppig and O'Brien, 1996; O'Brien et al, 2003).

1.11.6 Preantral Follicle culture

Although success has been achieved in the development of culture systems for rodents, findings from these systems cannot necessarily be applied to the domestic species and humans. Rodent systems are far less problematic in many respects however, in comparison to the domestic species and humans, as preantral follicle development in these species takes months and follicles also grow much larger. Although the culture of follicles from the domestic species is much more difficult, transferral of these systems from rodents is theoretically possible and their development is the aim of many research groups at the present time.

1.12 Domestic Species Preantral Follicle Culture

Success in the domestic species in harvesting healthy follicles and oocytes after culture has been less successful than in rodents. In the cow preantral follicles have been sustained for up to 28 days under serum-free conditions, but no histology was carried out on cultured follicles to assess health on termination of culture (Gutierrez et al, 2000). It was found in this study that follicle growth and antrum formation was enhanced by EGF, FSH and IGF-1. Preantral follicles have also been grown over a period of up to twelve days under serum-free conditions, with the addition of ascorbate (Thomas et al, 2001). In the culture of bovine antral follicles, it has been demonstrated that culture for 24 hours improves maturational rates of oocytes removed from cultured follicles in comparison to oocytes taken straight from the ovary (Fouladi Nashta et al, 1998). Follicle growth has also been initiated *in vitro* in the cow, when foetal ovarian tissue was cultured in a serum-free environment (Wandji et al, 1996).

In the sheep follicles which were manually dissected from ovarian tissue have been cultured for 6 days, with antral development (Cecconi et al, 1999). FSH was found to enhance follicle growth and stimulate antrum formation when cultured in 5% oxygen. Newton et al (1999) isolated follicles using collagenase and cultured them for 30 days.

Early experiments of porcine preantral follicle culture were carried out by Hirao et al (1994). Follicles of >200µm were isolated using a collagenase enzymatic treatment, and were cultured in the absence of a theca layer. Follicles were cultured in the presence of FSH, oestradiol, and foetal calf serum. Culture was terminated on day sixteen and oocytes were removed from follicles and matured *in vitro*. 40% of morphologically normal oocytes over the size of 110µm

underwent meiosis to metaphase II, but no oocytes progressed beyond this stage of meiosis. Sperm penetration was observed, but decondensation of the sperm head was incomplete, suggesting deficient cytoplasmic maturation of the oocytes. Oocytes from preantral follicles are meiotically incompetent, but Hirao had shown for the first time that oocytes were capable of acquiring the capacity to resume meiosis *in vitro*. Telfer et al (2000) cultured porcine follicles with similar results. Follicles in the size range 160-260µm were mechanically isolated and cultured for up to 20 days in the presence and absence of serum. Serum was found to significantly increase the percentage of follicles surviving the culture period. Oocyte cumulus complexes were removed from the follicle, and matured *in vitro*.

An apparent breakthrough in the culture of porcine preantral follicles was made in a study conducted by Wu et al (2001). Preantral follicles were isolated using forceps dissection and cultured in the presence of serum and FSH over four days, culturing follicles in groups of three. Follicles were grown for four days, and had their oocyte cumulus complexes removed after culture. 53% of fertilised oocytes cleaved to the blastocyst stage. This was a remarkable achievement as follicle and oocyte development to the fertilisable stage takes several months in the pig (Morbeck et al, 1992). These results have not yet been emulated, as preantral follicles cultured under similar conditions developed antral cavities, but oocyte maturation was not observed in cultured follicles using similar culture conditions (Mao et al, 2002).

A recent study by Shuttleworth et al (2002) showed porcine preantral follicles could be cultured for thirty days in groups of three in a collagen matrix under serum-free conditions. Follicles were isolated mechanically, and were cultured in

groups of three on collagen coated wells. After 30 days of culture follicle structure was found to be intact, and oocyte viability had been maintained. However, maturational status of the oocytes after culture was not ascertained.

1.13 Aims of Project

Great advances have been made in reproductive biology over the last two decades; however, further progress is hampered by a lack of availability of quality fertilisable oocytes. Techniques such as *in vitro* maturation (IVM) and cloning have the potential to improve animal production, but are hampered due to a lack of oocytes. Current IVM techniques have relatively low success rates, and this could be due to the utilisation of large oocytes taken from antral follicles. Follicles have a very high level of atresia at this stage, and are also highly heterogeneous, so using oocytes from these follicles is not ideal. In contrast, primordial and preantral follicles have very low levels of cell death, and if some way could be found to harvest the oocytes from such follicles, this could also prevent the high amounts of wastage seen *in vivo* and potentially provide a superior starting population of follicles for *in vitro* use.

The development of a culture system capable of producing large numbers of germ cells for use in *in vitro* techniques is a very long-term aim. The main aim of developing a culture system to support preantral follicles in the short term will be to try to elucidate requirements for growth and differentiation at each developmental stage. A serum-free culture provides an excellent model system to allow folliculogenesis to be characterised in a stage-dependent manner as specific elements can be added to medium to examine which factors are needed at each stage of development. The addition of supplements in a stage-dependent manner should allow the eventual characterisation of folliculogenesis, and aid our understanding of the complex requirements for successful oocyte and follicle development.

The development of a culture system capable of sustaining preantral follicles may also provide a means of identifying markers of follicle development. There is a major lack of understanding about early folliculogenesis, which could be partly overcome if markers of early stage follicles could be identified. Markers of development could be intra-ovarian regulators, such as activin or inhibin, or oocyte specific factors such as GDF-9. GDF-9 has been identified in several species, including rodents, cows, sheep, and humans (McGrath et al, 1995; Dong et al, 1996; Bodensteiner et al, 1999). GDF-9 has not yet been identified in the pig. The isolation and pattern of expression of GDF-9 could be useful as a potential marker of oocyte and follicle development.

The Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes which control extracellular matrix turnover. They have been implicated in follicle development at the latter stages (Puistola et al, 1986; Gottsch et al, 2002). MMP-9 in particular has been identified as a marker of preantral follicle health in cultured bovine follicles (McCaffery et al 2000). However, it has not been established if MMPs are involved in early porcine follicle development.

The main problems associated with the development of a culture system for large mammals in comparison to rodents are the large size of follicles once they are past the antral stage of development, and also the time-scale of follicle development from primordial through to ovulatory, taking months rather than weeks in mice. The development of a culture system capable of sustaining large mammalian follicles through from very early stages to a point where the oocyte can be removed and successfully fertilised *in vitro* therefore remains a distant reality. Rather, *in vitro* culture systems are limited to being a valuable experimental tool which should allow the characterisation of the stage-wise

requirements for follicle development, and also the identification of possible markers of development and health.

The main aims of this thesis therefore were to:

- (1) Identify factors affecting the development of porcine preantral follicles, and characterise the requirements for this stage of folliculogenesis using a serum free culture system.
- (2) Identify potential markers of porcine preantral follicle and oocyte development
 - (i) MMP production in culture medium as a possible marker of follicle health.
 - (ii) GDF-9 as a putative marker of oocyte and follicle development.

Chapter 2: General Materials & Methods

2.1 Materials and Methods

2.1.1 Preantral Follicle Isolation

2.1.1.1 Collection of Ovaries

Porcine prepubertal ovaries were collected from a local abattoir from pigs at unknown stages of the oestrous cycle, and transported to the laboratory within one hour. Ovaries were transported at 37°C in M199 [HEPES buffered medium supplemented with penicillin G (75µg/ml), and streptomycin (50µg/ml)]. Ovaries were then wiped with 70% ethanol to remove contamination, and dissected in a laminar flow hood using a scalpel. Ovary slices were prepared by slicing thin sections of the ovarian cortex, and the slices were then held in Leibovitz dissection medium (GIBCO BRL, Life Technologies Ltd., Paisley, Renfrewshire). All chemicals were from Sigma Chemicals, Poole, Dorset, UK, unless otherwise stated.

2.1.1.2 Dissection Medium

Ovary slices were held in Leibovitz medium, and subsequent follicle dissection work was also carried out in this medium. Leibovitz was supplemented with penicillin G (75µg/ml), streptomycin (50 µg/ml), glutamine (2mM), sodium pyruvate (2mM), and BSA (3mg/ml). Before use in tissue manipulation, the Leibovitz medium was incubated at 37°C.

2.1.1.3 Follicle Dissection

Preantral follicles (175-275µm) were isolated from cortical strips using fine 25G needles (Merck Ltd., Lutterworth, UK) attached to syringe barrels under a dissecting microscope (Olympus, UK) which was fitted with a calibrated eyepiece graticule (Graticules Ltd., Tonbridge, Kent, UK). Follicles were selected on the

basis they had an intact basement membrane, even granulosa and theca layers, and no sign of antrum formation (see figure 2.1; figure 2.2).

2.1.2 Preantral Follicle Culture

2.1.2.1 Culture Medium

Follicles were cultured in either McCoys medium (GIBCO BRL, Life Technologies Ltd, Paisley, Renfrewshire, UK) or North Carolina State University (NCSU) 23 medium. McCoys 5a medium with bicarbonate and HEPES (20mM) was prepared and supplemented with bovine serum albumin (BSA; 0.1%), L-glutamine (3mM), penicillin (100IU/ml), streptomycin (0.1mg/ml) transferrin (2.5µg/ml), selenium (4ng/ml), androstenedione (10^{-7} M) and insulin (10ng/ml). Medium was filter sterilised using syringes (Merck) and filters (Corning Costar, UK), and incubated at 37°C. Medium was supplemented with either 50µg/ml of ascorbate, 2miu/ml porcine pituitary FSH, or 10% fetal bovine serum. Ascorbate was added fresh every two days, as it is very unstable in culture, and is rapidly oxidized to dehydroascorbic acid (Padh and Aleo, 1985).

NCSU 23 medium (for composition of NCSU see appendix 1) was supplemented with 1.5 ng/ml FSH (NIDDK-oFSH-20), 7.5% porcine serum, and 3 mg/ml BSA. The culture medium consisted of NCSU 23 supplemented with 3.5 µg/ml insulin, 10 µg/ml transferrin, and 100 µg/ml ascorbate. All chemicals from Sigma, Poole, Dorset, UK, unless stated otherwise.

2.1.2.2 *Preantral Follicle Culture*

Preantral follicles were cultured individually in McCoys medium in 96 well plates (Bibby Sterilin Ltd., Stone, Staffs, UK) in 250 μ l (follicles in individual culture) and 500 μ l of medium (groups) with the appropriate treatment. Plates were cultured for 6 days in a sterile humidified atmosphere with 5% CO₂ at 37°C. Half of the culture medium was replaced every two days with fresh medium. Medium removed was stored at -20°C for subsequent MMP analysis. Follicle diameters were measured every two days, on days 0, 2, 4, and 6. They were measured on two planes, and an average of the two measurements was recorded.

2.1.3 Histology

2.1.3.1 Fixation of Tissue

On termination of culture follicles were fixed for four hours in 4% paraformaldehyde (4% paraformaldehyde in dH₂O, 0.1M NaOH, 0.1M Na phosphate pH 7, 4°C) or in Bouins fixative solution (70% picric acid, 25% formaldehyde, and 5% glacial acetic acid). Whole ovary slices were fixed in paraformaldehyde overnight for use in *in situ* hybridization experiments. Fixative was replaced with 70% ethanol treated with eosin (Merck), to aid follicle visualization during processing.

2.1.3.2 Sample Processing

Follicles were dehydrated through a series of graded alcohol changes, up to absolute ethanol. 100% alcohol was then removed, and replaced with cedar wood oil (Merck) for at least 24 hours. All traces of oil were then removed using toluene. Follicles were left in toluene for 30 minutes, and then embedded in paraffin wax (Merck) at 60°C. Wax was changed every hour for four hours to remove toluene.

2.1.3.3 Sectioning and Mounting

Wax samples were sectioned using a microtome (Leica UK Ltd., Milton Keynes, UK) and were cut at 6µm intervals. Sections were then floated out in a water bath at 42°C onto gelatin coated slides. Sections for use in *in situ* hybridization experiments were mounted on charged slides.

2.1.3.4 Staining

Sections were dewaxed in a xylene bath for 15 minutes and rehydrated through alcohols from 100% to 70%. Slides were then dipped in 70% lithium carbonate to

remove Bouins, and were then rinsed in tap water. Reagents for staining were made up according to the protocol by Drury and Wallington (1976). Slides were then placed in Harris' haematoxylin (BDH chemicals, Poole, Dorset, UK) for 5 minutes, then rinsed in water and put in Scott's tap water substitute (see appendix 2 for composition of Scott's tap water substitute). Sections were rinsed again in tap water, and put in eosin (Merck) for 2 minutes. Slides were then dipped quickly in water and put in potassium alum (Merck) for 3 minutes. Sections were then dehydrated through a series of graded alcohols from 70% to absolute and placed in xylene. Sections were finally mounted using p-Xylene-bis(N-pyridinium bromide) (DPX) mounting medium (Merck), with a glass coverslip on top of sections.

2.1.3.5 Collection of Histological Results

Histological measurements were made under the light microscope. The section containing the germinal vesicle oocyte was used for measurements and observations. Oocyte size was measured on the light microscope using a crossed micrometer (Graticules Ltd.). Granulosa cell death was measured by counting the total number of pycnotic nuclei present in the section of the follicle containing the oocyte, and comparing the number to healthy cells. At least 150 cells were counted for each follicle, and a consistent level of measurement was maintained throughout all treatments. The number of healthy cells compared to atretic granulosa cells was expressed as a percentage, and follicles displaying >5% pycnosis were classified as atretic (Byskov, 1974). Oocytes were considered healthy if they were round and not misshapen, and contained an intact GV. Theca cell health was measured in the

same way as granulosa cells, by counting the percentage of pycnotic to normal cells. Antral cavity formation was also noted.

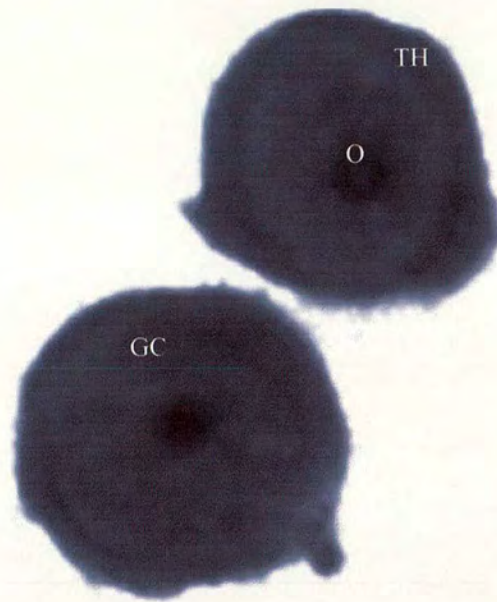


Figure 2.1: Freshly Isolated Follicles: Porcine preantral follicles obtained by micro-dissection. The basement membrane is intact, granulosa layers (GC) are even, and the oocyte is visible (O). Theca (TH) tissue can also be seen round the outer surface of the follicles. Bar represents 50 μ m.

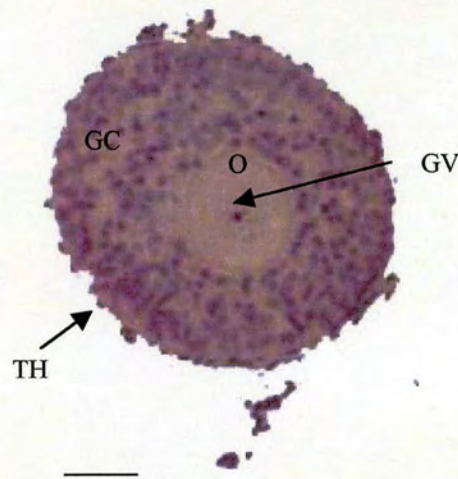


Figure 2.2: Freshly Isolated Follicle Fixed for Histology. Porcine preantral follicle obtained by micro-dissection, fixed, serially sectioned, and stained with haematoxylin and eosin. The basement membrane is intact, granulosa layers (GC) are even, and the oocyte is visible (O) with an intact germinal vesicle (GV). Thin theca (TH) tissue can also be seen round the outer surface of the follicle. Bar represents 50 μ m.

**Chapter 3: The Effects of Ascorbate, Follicle Stimulating
Hormone and Serum on the Development of Porcine
Preantral Follicles *in Vitro***

3.1 Introduction

The isolation and culture of mammalian preantral follicles provides a useful tool to study early folliculogenesis, and identify the key factors which control this process. The culture of preantral follicles has been achieved with varying levels of success, as has been previously discussed in section 1.10-1.12. Many important regulators of folliculogenesis have been identified using *in vitro* culture systems, including growth factors, locally produced factors, serum, and ascorbate (Nayudu et al, 2001). The addition of serum to medium is beneficial to follicle growth and development, but is not ideal as it introduces undefined elements into the medium, and therefore specific effects on follicle development cannot be attributed to factors which are added to medium. For this experiment it was decided to compare serum and serum-free medium, and to test the effects of ascorbate and FSH using a serum-free medium.

Ascorbate has been associated with many diverse functions within the body, including cancer (Padayatta and Levine, 2001), ageing and Alzheimer's disease (Martin, 2003), and reproductive function (Luck et al, 1995). The effects of ascorbate on early follicle development have been studied in the cow (Thomas et al, 2001) and the mouse (Murray et al, 2001). Culture of preantral follicles in these species showed that the addition of ascorbate to culture medium resulted in a decrease in somatic cell death, and also an increase in the percentage of follicles maintaining an intact basement membrane throughout the culture period. A similar effect has been shown in cultured rat antral follicles (Tilly and Tilly, 1995). It is postulated that ascorbate exerts an anti-apoptotic effect on somatic cells by disposing of free radicals when it is oxidised (Padh et al, 1991). This experiment was carried out to ascertain whether or not a similar anti-

apoptotic effect would be observed in cultured porcine preantral follicles, and also whether it would have a positive effect on basement membrane integrity. Although much has been documented about the anti-oxidant properties of ascorbate, concerns have been raised by research demonstrating its ability to act as a pro-oxidant at high levels. It has been proven to be an anti-oxidant at low levels, but conversely it displays pro-oxidant properties at high concentrations (Griffiths and Lunec, 2001). Thus great care must be taken in selecting an appropriate dose.

Follicle stimulating hormone (FSH) is a principal regulator of ovarian function, and is essential for normal follicle development beyond the preantral stage (Hisaw, 1947; Kumar et al, 1997; Dierich et al, 1998; Burns et al, 2001). FSH is a principle regulator of antral follicle development, but its role in the development of preantral follicles remains largely undefined. It has been proved that FSH is not an absolute prerequisite for preantral follicles, as animals which have had their pituitary gland removed (hypophysectomy) produce growing follicles, and these follicles can reach the preantral stage. However, follicles become atretic when they develop an antrum in the absence of FSH in hypophysectomised animals, thus highlighting the essential nature of FSH in antral follicles (Hisaw, 1947). The treatment of hypophysectomised rats with FSH reverses the effects of hypophysectomy, and antral follicle development is observed, along with an increase in ovary weight (McGee et al, 1997b).

Further studies looking at the effects of FSH have been carried out using mice which have mutations in the FSH gene. Mice which have the gene knocked out for the FSH β gene are incapable of producing follicles beyond the preantral stage of development (Kumar et al, 1997; Burns et al, 2001). These mice have

low levels of circulating FSH, and have increased FSH receptor (FSHR) mRNA expression. Mice which have mutations in the FSHR gene produce primordial, primary and secondary follicles, but no mature follicles, and are also infertile (Dierich et al, 1998).

The expression of the FSH receptor was thought to be limited to the granulosa cells of the follicle. Many experiments in different species have shown that FSH receptors are present only in the granulosa cells of the follicle, including sheep (Tisdall et al, 1995; Eckery et al, 1997), cow (Wandji et al, 1992), and rat (Monniaux and De Reviers, 1989; Tilly et al, 1992a; Rannikki et al, 1995). In the bovine foetus granulosa cell expression of the FSH receptor is found to increase significantly from the primary transition through to antral stages, although the receptor is present in primary follicles (Wandji et al, 1992).

However, a recent study found the presence of FSH receptor mRNA in human oocytes (Meduri et al, 2002), raising doubts on whether FSH receptors could also be present in the oocyte. In the pig it has been found that FSH receptors are expressed mainly in the granulosa compartment of the follicle (Yuan et al, 1996; Liu et al, 2000; Slomczynska et al, 2001). FSHR expression appears to be present from antral stages onward, and this expression decreases as the follicles grow larger (Liu et al, 2000; Cardenas and Pope, 2002), although further work is required to determine exactly what stage of follicle development FSHR expression begins in the porcine.

The importance of FSH as a principal regulator of antral follicle development has been identified, but its role in preantral development is less well defined. FSH has been found to have a beneficial, stimulatory effect on the development of preantral follicles in several species. *In vitro* studies have identified a role for

FSH in oocyte development and growth (pig; Hirao et al, 1994), follicle growth (sheep; Cecconi et al, 1999), granulosa cell health and proliferation (mouse: Cortvrindt et al 1997), and steroid production (sheep; Campbell et al, 1996; Cecconi et al, 1999). FSH has also been shown to have effects on preantral follicle development *in vivo*, as pregnant mare serum gonadotrophin (PMSG) treatment of unilaterally ovariectomied heifers increased the number of preantral follicles present in the remaining ovaries, and also increased granulosa cell proliferation (Monniaux et al, 1984).

The aim of the present experiment was to test the effects of FSH *in vitro* in this culture system and to identify the effects of FSH on whole porcine preantral follicles. The main experiments which have been carried out to date on early follicles from the pig have involved enzymatic dissection of follicles, so these follicles do not have an intact basement membrane, and therefore may not give an accurate representation of actual events in folliculogenesis as the theca layer is not present (Hirao et al, 1994). The results from these experiments showed that FSH enhanced oestradiol production, induced follicle growth and decreased follicle cell death. The present experiment was designed to test the effects of FSH using whole follicle culture, and culture them individually so follicles could not be exerting an effect on each other.

The main aims of the present experiment were therefore to:

- (1) Examine the potential anti-apoptotic effects of ascorbate in the culture of porcine preantral follicles.
- (2) Identify a role for FSH in preantral follicle development.
- (3) Identify effects of serum on preantral follicles *in vitro* compared to under serum-free conditions.

3.2 Materials and Methods

3.2.1 Follicle Isolation and Culture

Preantral follicles of size 175-275 μ m were isolated from fresh porcine ovaries as described in section 2.1.1. They were cultured for six days individually in 96-well plates (Corning Costar, 3894) in McCoys serum free 5A medium modified with 25MM HEPES buffer (SIGMA chemicals; Poole, Dorset, UK). Follicle stimulating hormone and foetal bovine serum were also purchased from SIGMA. Follicles which were not fixed for histology after six days of culture were removed and burst using fine needles. The oocytes from these follicles were collected using a finely drawn glass capillary tube, and placed on a slide. A glass cover-slip was placed over the oocytes, and oocytes were then stained using 1% aceto-orcein stain (SIGMA), and observed under the microscope. The number of attached cumulus cells were then counted and recorded.

3.2.2 Treatment Groups

Follicles were cultured in McCoys medium, prepared as described in chapter 2.

The treatment groups used were:

- (1) control
- (2) ascorbate (50 μ g/ml)
- (3) FSH (2miu/ml)
- (4) FSH (2miu/ml) & ascorbate (50 μ g/ml)
- (5) Serum (10%) & ascorbate (50 μ g/ml)

3.2.3 Statistical Analysis

Follicle growth was compared using the Kruskal-Wallis test among all treatment groups, and a Mann-Whitney comparison was used to compare between individual treatment effects. The same tests were used to determine the differences of treatment effect on oocyte size, number of theca cell layers after culture and the attachment of cumulus cells to the oocyte after six days of culture. Follicles which displayed >5% granulosa cell pycnosis were considered to be atretic, and these data were analysed using Fisher's Exact test. All other parameters of follicle health and development were also compared using the Fisher's Exact test. P values of <0.05 were considered to be significant.

3.3 Results

3.3.1 The Effects of Ascorbate, FSH and Serum on Preantral Follicle Growth

Follicle growth was significantly greater in medium cultured in the presence of FSH and FSH with ascorbate until day 4 of culture, after which time the difference in mean follicle diameter was not significantly different in comparison to controls (FSH: day 2 $p=0.01$; day 4 $p=0.015$; day 6 $p=0.19$; FSH with ascorbate: day 2 $p=0.023$; day 4 $p=0.038$; day 6 $p=0.24$). Ascorbate had no effect on growth compared to FSH treated follicles on all days of culture (day 2, $p=0.0017$; day 4, $p=0.0006$; day 6, $p=0.0056$). Follicles also grew significantly more in the presence of ascorbate with FSH over all days of culture (day 2 $p=0.0077$; day 4 $p=0.001$; day 6 $p=0.018$) compared to ascorbate alone. Follicle growth was not different between serum with ascorbate follicles and FSH-treated follicles (day 2, $p=0.57$; day 4, $p=0.99$; day 6, $p=0.34$). Follicle growth was also not significantly different between follicles cultured in serum with ascorbate and FSH with ascorbate (day 2, $p=0.89$; day 4, $p=0.93$; day 6, $p=0.73$). There was no difference between ascorbate and control follicles on any day of culture. These results are illustrated in figure 3.3.1.

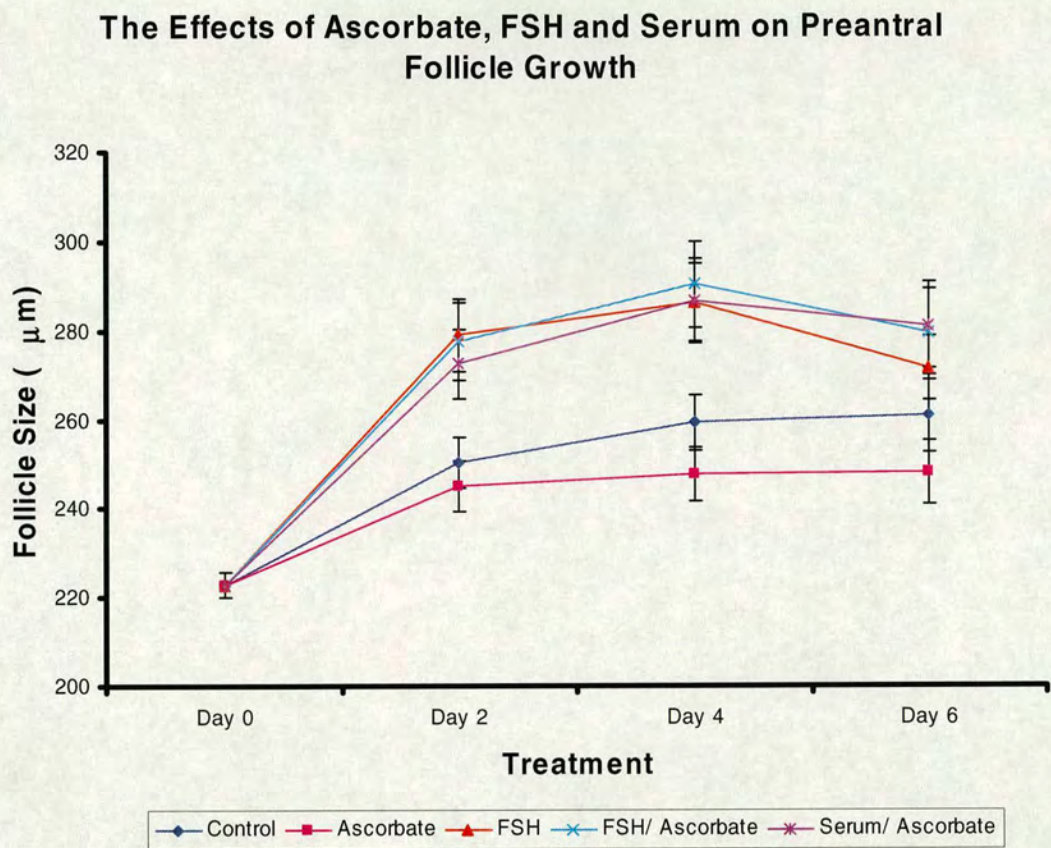


Figure 3.3.1 The Effects of Ascorbate, FSH and Serum on Preantral Follicle Growth. The growth of preantral follicles over six days in control medium (navy blue, n=75), ascorbate (pink, n=74), FSH (red, n=66), FSH with ascorbate (light blue, n=70), and serum with ascorbate (purple, n=74) is demonstrated in figure 3.3.1. Overall p value day 2 p=0.003; day 4 p=0.000; day 6 p=0.016. Values are mean± SEM.

3.3.2 *The Effects of Ascorbate, FSH and Serum on Follicle Atresia*

Follicles which were cultured in control medium had a significantly greater number of follicles displaying more than 5 % pycnosis (i.e. classified as atretic) than follicles which were cultured in medium supplemented with ascorbate ($p=0.016$). There were no other differences between treatment groups. The percentage of follicles displaying >5% pycnotic granulosa cells is represented in figure 3.3.2.

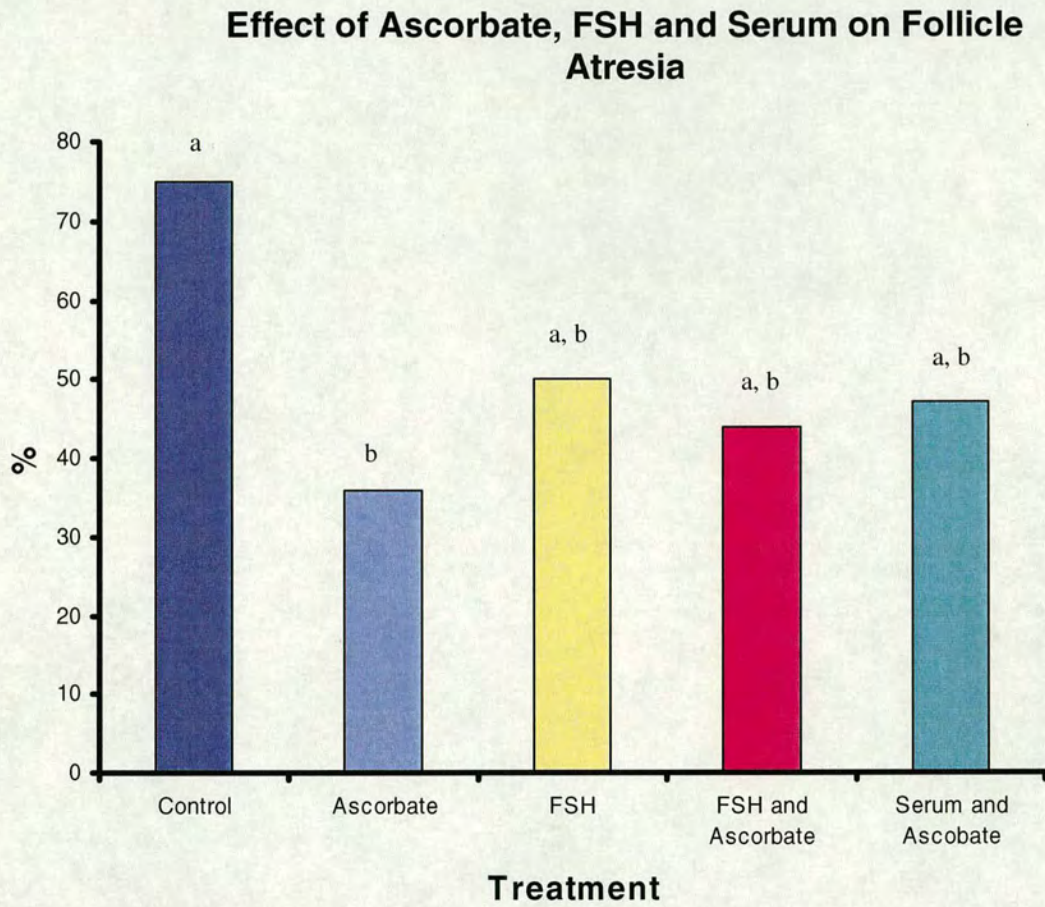


Figure 3.3.2 Effect of Ascorbate, FSH and Serum on Follicle Atresia. Control follicles (navy blue, n=20) had significantly more atretic follicles than ascorbate (purple, n=22). FSH follicles (yellow, n=26), serum with ascorbate (light blue, n=16) and FSH and ascorbate treated follicles (pink, n=19) displayed no significant differences in comparison to other treatment groups. Values are percentage of atretic follicles (>5% pycnotic granulosa cells) per treatment group. Different letter denotes significant difference in raw data.

3.3.3 Histological Examination of Cultured Follicles

Histological examination of cultured follicles revealed that there were no significant differences between any of the parameters measured, except the number of atretic follicles cultured with ascorbate in comparison to controls ($p=0.016$) and the number of follicles maintaining an intact basement membrane between serum and ascorbate treated follicles and FSH-treated follicles ($p=0.046$). There were no differences between the number of theca layers in the different groups, oocyte degeneration, theca degeneration, antrum formation and integrity of basement membrane except the difference in basement membrane integrity, as mentioned above (see table 3.3.1).

Table 3.3.1 :The Effects of Ascorbate, FSH and Serum on Follicle Histology. The effects of ascorbate, serum and FSH treatment on oocyte diameter, number of theca layers, oocyte degeneration, granulosa cell degeneration, theca degeneration, antrum formation, and basement membrane integrity are displayed in table 3.3.1.

Treatment	Mean		Mean No.		Oocyte		Granulosa		Theca		Antrum		Basement	
	Oocyte		Theca		Degeneration		Degeneration		Degeneration		Formation		Membrane	
	Diameter ($\mu\text{m}\pm\text{SEM}$) *		Layers ($\pm\text{SEM}$)		(%)		(% follicles >5% Pycnotic GC)		(%)		(%)		Intact (%)	
Control (n=20)	66.4 ± 3.2		2.4 ± 0.2		33.3		75		90		16.6		83.3 ^{a,b}	
Ascorbate (n=22)	64.0 ± 3.4		2.8 ± 0.3		20		36.4		80		36		80 ^{a,b}	
FSH (n=26)	54.8 ± 2.4		1.9 ± 0.2		50		50		62.9		22.2		62.9 ^a	
FSH & Ascorbate (n=19)	60.5 ± 5.1		2.1 ± 0.2		50		44.4		89.5		29.4		89.5 ^{a,b}	
Serum & Ascorbate (n=16)	69.7 ± 3.9		2.1 ± 0.3		42		47.0		90		15.8		90 ^b	
Overall p value	p=0.099		p=0.16											

* $\pm\text{SEM}$: \pm Standard error of the mean. Different letter denotes significant difference.

3.3.4 Follicle Morphology



Figure 3.3.3 (a)

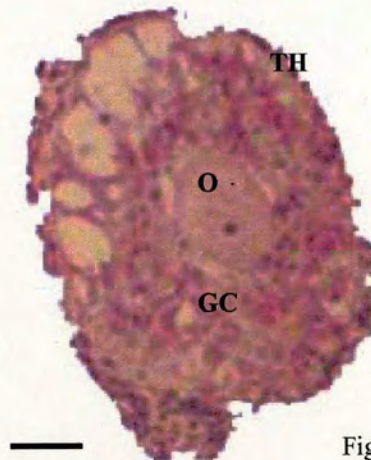


Figure 3.3.3 (b)

Figure 3.3.3(a) and 3.3.3(b): Examples of six day cultured follicles. Figure 3.3.3(a) is a healthy follicle with an antral cavity. The oocyte is round and healthy and the germinal vesicle can be observed. There are several theca layers surrounding the follicle. Figure 3.3.3(b) is an example of a heavily atretic follicle. Degeneration of the somatic cells is apparent with wide-spread pycnosis. The oocyte looks relatively healthy however, and the germinal vesicle can be observed. Bar represents 50 μ m. (O=oocyte; GC=granulosa cells; TH=theca cells).

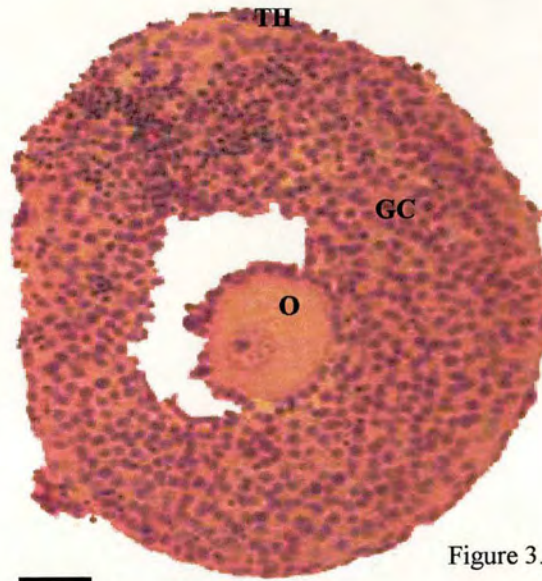


Figure 3.3.3 (c)

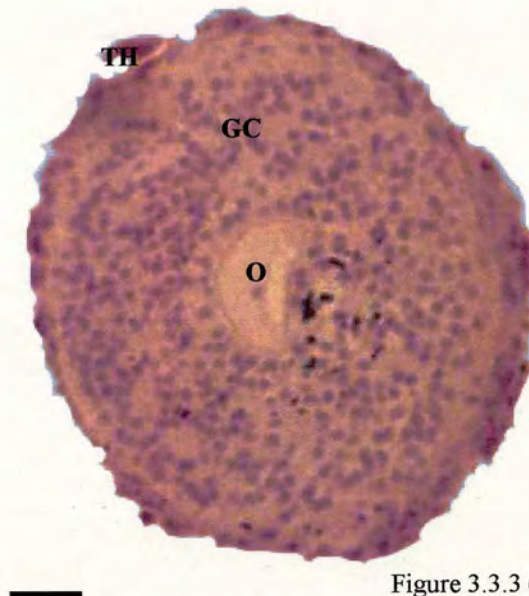


Figure 3.3.3 (d)

Figure 3.3.3(c) and 3.3.3(d): Examples of six day cultured follicles. Figure 3.3.3(c) is a healthy follicle with an antral cavity. The oocyte is round and healthy and the germinal vesicle can be observed. There are no theca layers present. Figure 3.3.3(d) is an example of a relatively healthy follicle with a degenerate oocyte and some localised pycnosis close to the oocyte. The rest of the granulosa cells and the theca cells appear healthy. Bar represents 50 μ m. (O=oocyte; GC=granulosa cells; TH=theca cells).

3.3.5 The Effects of Ascorbate, FSH and Serum on Cumulus-Oocyte Contact

The mean number of cumulus cells attached to the oocyte after six days of culture is displayed in figure 3.3.4. Ascorbate was found to have a positive effect on the number of cumulus cells present after six days of culture as there were significantly more cumulus cells attached to the oocyte compared to controls (n=8) after culture in the presence of ascorbate (n=14; $p=0.014$) and also in comparison to follicles cultured with FSH alone (n=11; $p=0.0003$). However, a significant difference was not seen for follicles cultured in the presence of serum with ascorbate (n=7; $p=0.81$) or FSH with ascorbate (n=8; $p=0.10$) compared to controls. Ascorbate alone also had a positive effect on cumulus oocyte contact in comparison to serum with ascorbate ($p=0.0072$), and FSH with ascorbate treated oocytes retained contact with cumulus cells significantly better than those cultured in FSH alone ($p=0.01$).

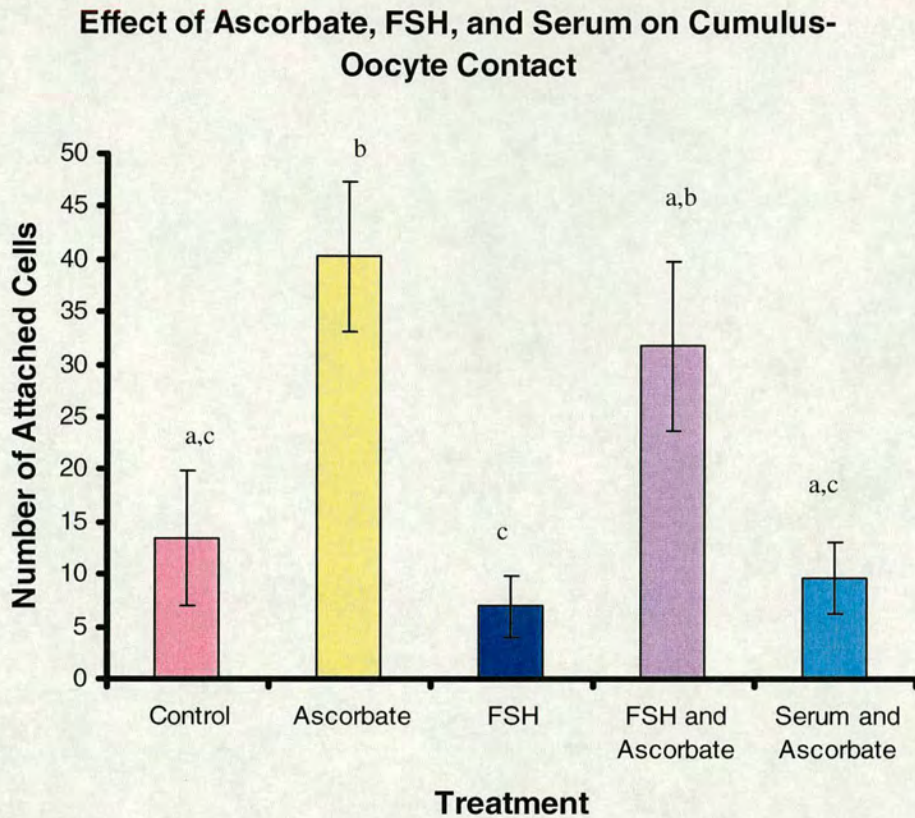


Figure 3.3.4: Effect of Ascorbate, FSH and Serum on Cumulus-Oocyte Contact. There were significantly more cumulus cells attached to the oocyte in the presence of ascorbate (yellow, $n=14$) compared to controls (pink, $n=8$) and FSH follicles which were cultured with ascorbate (purple, $n=8$). The difference between serum with ascorbate treated follicles (light blue, $n=7$) was also not significantly different than controls or FSH (dark blue, $n=11$). Overall p value comparing all treatment groups $p=0.001$. Results are \pm SEM. Different letter denotes significant difference ($p<0.05$).

3.4 Discussion

The effects of ascorbate, FSH, FSH with ascorbate, and serum with ascorbate have been studied in the present experiment. It was found that the addition of FSH, FSH with ascorbate, and serum with ascorbate significantly increased growth in comparison to control follicles at days 2 and 4 of culture, but not at day 6. However, it was also found that the addition of FSH, FSH with ascorbate, and serum with ascorbate significantly increased growth of follicles in comparison to follicles treated with ascorbate alone, and this difference was found to be significant up to day 6. Ascorbate was found to have a positive effect on granulosa cell survival in culture, as there were significantly less atretic follicles in this group in comparison to control follicles. Ascorbate was also found to have a positive effect on somatic cell contact between the granulosa cells and oocyte. There were significantly more attached cumulus cells after 6 days of culture than those cultured in the presence of ascorbate in comparison to controls, FSH, FSH and ascorbate and serum with ascorbate. The results from this experiment indicate that ascorbate has no effect on the growth of preantral follicles *in vitro*. This is consistent with previous studies which showed that ascorbate had no effect on preantral follicle growth in the mouse (Murray et al, 2001) and the cow (Thomas et al, 2001). Although ascorbate had no effect on follicle growth, FSH and serum were found to significantly increase growth until day 4 in comparison to controls, and day 6 compared to ascorbate. FSH and serum treatment resulted in significantly greater follicle growth than in ascorbate follicles on all days of culture, indicating that addition of these factors does stimulate follicle growth and development. It also implies that FSH receptors are present in follicles at the

preantral stage of development, as FSH increased growth in this system, which is consistent with other findings in the pig (Hirao et al, 1994). However, this effect may not be beneficial to follicle development as after day four follicle size growth rates appeared to slow down in FSH and serum-treated follicles. This could imply that initial gonadotrophin treatment results in an initial stimulatory response, but this response does not continue due to a desensitization of the receptors. This effect is observed in women being treated for infertility.

Administration of exogenous gonadotrophins leads to a stimulatory response, but the gonadotrophic cells then become desensitized and this leads to a down-regulation of the receptors (Reissman et al, 1995). A similar effect may have occurred in the experiment described in the present chapter, where FSH had an initial effect on follicle growth, but due to down-regulation of the receptors this effect was not observed after day 4, and also may have resulted in premature luteinization due to over-stimulation. However, ascorbate treated follicles and control follicles appeared to be growing at a more constant rate.

Histological examination of follicles revealed that FSH was not a survival factor in this culture system, as it did not decrease atresia in follicles compared to controls. In comparison, granulosa cell death was significantly reduced in ascorbate treated follicles compared to controls, as has been found in the mouse (Murray et al, 2001) and cow (Thomas et al, 2001). Ascorbate is a known anti-oxidant which exerts an effect by taking in free radicals, and this is presumably the mechanism by which it reduces cell death in granulosa cells in cultured preantral follicles. The role of FSH as a survival factor in preantral follicles remains unresolved. It is claimed by some that FSH acts as a survival factor in preantral follicles although this view is controversial. There is no doubt that

FSH acts on preantral follicles, as it stimulates growth in cow (Gutierrez et al, 2000; Itoh et al, 2002), sheep (Cecconi et al, 1999), mice (Cortvrindt et al, 1997), and pig (Hirao et al, 1994), and the present study. It has also been found to promote antrum formation and follicle differentiation in the afore-mentioned studies. However, experiments on cultured rat preantral follicles have shown that addition of FSH, or its downstream mediator cAMP, to serum-free medium does not reduce DNA fragmentation patterns (McGee et al, 1997a). This suggests that FSH does act on preantral follicles, but may not necessarily be a useful addition to culture medium as it may not suppress follicle apoptosis. Eppig et al (1998) found a deleterious effect of FSH on the developmental competence of oocytes which were cultured in the presence of insulin. FSH and insulin were found to have a beneficial effect on oocyte development individually, but together they had an extremely deleterious effect. It was postulated in this paper this effect could have been due to insulin and FSH acting synergistically to cause inappropriate granulosa cell differentiation. Insulin and FSH could have acted in this manner in the present system to cause precocious and inappropriate granulosa differentiation, resulting in the lack of a survival effect on granulosa cells which was observed here. However the concentration of insulin used in the Eppig study was far higher (5 μ g/ml) than the close to physiological concentration used in this study (5ng/ml). The physiological concentration of insulin in porcine follicular fluid is 12.8 μ IU/ml (Quesnel et al, 2000), which converts to 0.53ng/ml (LabQuest Biochemistry Conversion webpage). To examine the specific role of FSH in this system, the experiments could be repeated without the addition of insulin to the medium.

This should give a more accurate representation of the true effects of FSH on the development of porcine preantral follicles *in vitro*.

Serum is a known survival factor for preantral follicles, but is not ideal as it introduces undefined elements into medium, making it difficult to assess effects of other factors added to medium. In the present experiment serum with ascorbate significantly increased follicle growth compared to ascorbate, but there was no difference compared to FSH treated follicles. Serum with ascorbate did not significantly reduce granulosa cell death in comparison to controls.

The maintenance of oocyte-cumulus contact was also measured in the present experiment. It was found that the addition of ascorbate to medium appeared to maintain cumulus-oocyte contact better than control follicles. Oocyte-cumulus contact is maintained by gap junctional contacts (Anderson and Albertini, 1976; Wright et al, 2001), and this contact is vital for oocyte development as it maintains meiotic arrest and provides essential ions and nutrients (Eppig, 1991). The resumption of oocyte maturation is also controlled by cAMP, and it has been postulated that ascorbate actions are regulated by cAMP. Experiments have been carried out involving the treatment of oocyte cumulus complexes and denuded oocytes with ascorbate *in vitro* (Guarnaccia et al, 2000). Ascorbate uptake is only observed in intact oocyte-cumulus complexes, indicating that cumulus cells mediate ascorbate action in the oocyte. The results from this experiment indicate that treatment with ascorbate of preantral porcine follicles helps to maintain the connections between the oocyte and surrounding cumulus cells. This is consistent with other studies, which have shown that inhibition of gap junctional contacts in smooth muscle cells by tumor necrosis factor- α (TNF- α) can be reversed with ascorbate treatment, suggesting it is an important

regulator of gap junctional contacts (Mensink et al, 1995). It seems possible that ascorbate may help to maintain cumulus-oocyte contact through enhancing trans-zonal projectional contact. One puzzling observation from this experiment was that serum with ascorbate did not have a positive effect on oocyte-cumulus contact, even though ascorbate was present in the medium. It is possible that if the serum contained LH, or something similar, it could have prevented the oocyte from uptake of ascorbate, thus preventing any positive effect it may have had on oocyte-cumulus contact. The serum was from the bovine; however, the effects of bovine gonadotrophins on porcine follicles have not been identified, and it is not known how species-specific they may be. However, *in vivo* studies have shown that porcine FSH can be used to superovulate cattle (Sugano et al, 2001), and pigs are treated with pregnant mare serum gonadotrophin and human chorionic gonadotrophin in superovulation (Ratky et al, 2001). It therefore seems possible that bovine gonadotrophins could have had an effect on porcine receptors in this system. FSH did not appear to have a positive effect on maintaining somatic cell and oocyte contact either. It is possible FSH has a similar effect to LH on oocyte uptake of ascorbate, as LH has been shown to deplete ascorbate from preovulatory follicles (Guarnaccia et al, 2000).

In conclusion, the results from this chapter indicate that ascorbate has a positive effect on porcine preantral follicle development *in vitro* as it reduced follicle atresia, and maintained oocyte-cumulus cell contact throughout culture. FSH and serum enhanced follicle growth during culture, but did not have a positive effect on somatic cell death or cumulus oocyte contact. Serum with ascorbate did not reduce granulosa cell death, and did not have a positive effect on cumulus oocyte contact, in comparison to controls.

**Chapter 4: Porcine Preantral Follicle Interactions *in Vitro*,
and the Effect of Different Culture Wells and Serum on
Development**

4.1 Introduction

The effects of follicle co-culture have been studied in several species using *in vitro* culture systems. Most of this work has been carried out using the mouse as a model system for the domestic species and humans (Spears et al, 1996; Baker et al, 2001). Porcine follicles have been cultured successfully in-groups of five in a collagen matrix, but no effects of follicle interactions were examined in this study (Hirao et al, 1994). A recent publication however, described an *in vitro* culture system for porcine preantral follicles that could produce oocytes capable of reaching the blastocyst stage of development. The optimum conditions of culture were found to be when follicles were cultured in-groups of three, in comparison to individually or in-groups of five (Wu et al, 2001).

Follicle co-culture systems in the mouse have found that the presence of other follicles has a negative effect on the development of some follicles, causing them to become subordinate, while one follicle becomes dominant. When two similarly sized follicles are cultured together one becomes dominant and the other subordinate. However this effect is normally only observed if follicles are cultured in direct contact with each other (Spears et al, 1996). It was deduced from this mouse study that any secretory factors produced by follicles in culture are non-diffusible in culture medium, as inhibitory effects were only observed when follicles were in direct contact with one another. In the system devised by Wu et al (2001), it was found that follicles grew best when cultured in-groups of three, in comparison to being cultured either individually or in-groups of five. Presumably any stimulatory effect observed from follicle co-culture must have come from a

diffusible factor, and this was postulated to be growth factor regulation. After four days of culture 68% of healthy cumulus oocyte complexes were obtained, and 51% of these oocytes completed meiotic maturation to the metaphase II stage. 53% of mature oocytes underwent fertilisation, 43% cleaved and 13% got to the blastocyst stage. This was an incredibly high rate of development for oocytes taken from preantral follicles, after only four days in culture. The present study was undertaken to examine the repeatability of this experiment, and also to look at other parameters of oocyte and follicle health using histology, something which was not examined by the Wu group. The experiment was repeated by Mao et al (2002), but they did not achieve the results obtained by Wu et al (2001). None of the oocytes recovered from the cultured follicles in the Mao experiment (2002) were able to progress to metaphase II.

The aims of this experiment therefore were to identify the effects of co-culture on follicle development, by culturing follicles individually or in groups of three, and also to examine the effects of using different culture plates on the development of preantral follicles. The study was also undertaken to determine the repeatability of the study carried out by Wu et al (2001) as that study showed tremendous results in a very short period of time.

4.2 Materials and Methods

4.2.1 Collection of Ovaries

Porcine prepubertal ovaries were collected as described in general material and methods section (2.1), and transported to the lab in M199 medium within one hour of sacrifice. They were rinsed in 70% alcohol to remove contamination, and cut into thin slices. The slices were stored in Leibovitz dissection medium until required.

4.2.2 Follicle Dissection

Ovary slices in Leibovitz medium were placed under the dissecting microscope in a glass petri dish. Follicle dissection was done using fine 25G needles, as described previously (section 2.1). Follicles of size 175-275 μ m were selected on the basis that they had an intact basement membrane, even granulosa and theca cell layers, and no sign of antrum formation.

4.2.3 Follicle Culture Medium

Follicles were cultured in North Carolina State University 23 medium (see appendix 1 for composition of NCSU 23). Medium was supplemented with 3.5 μ g/ml insulin; 10 μ g/ml transferrin; 100 μ g/ml ascorbate, 1.5ng/ml ovine FSH (NIDDK-oFSH-20) and 7.5% porcine serum. Groups which were cultured with serum contained ascorbate and FSH. Follicles cultured in control medium did not have the addition of serum, FSH or ascorbate. Follicles were either cultured in groups of three in 24-well culture plates, or individually in 96-well plates (Corning). Follicles were cultured in 250 μ l of medium in 96-well plates and 500 μ l in 24-well plates. Follicles were cultured for 4 days, and measured on days 0, 2 and

4. Half of the medium was replaced every two days. Follicles were fixed for histological examination after four days, and processed as described in materials and methods section 2.1.3. The 6 μ m follicle sections were then analysed to look at somatic cell degeneration, antrum formation, and oocyte quality and size. As before, all chemicals from SIGMA, Poole, Dorset, unless stated otherwise.

4.2.4 Statistical Analysis

The data from this experiment were analysed using the Kruskal-Wallis test, Mann-Whitney test, and Fisher's Exact Test. Follicle growth differences were compared overall using the Kruskal-Wallis comparison, and if a significant difference was observed among treatment groups, individual treatments effects were then compared using the Mann-Whitney test. Oocyte size and number of theca layers were also compared in this way. All other parameters of oocyte and follicle health and development were compared using the Fisher's Exact test. Differences were considered to be significant if they had a p value of <0.05.

4.3 Results

4.3.1 Effects of Co-Culture and Serum on Follicle Growth

Follicle growth rate was greatest when follicles were cultured individually in the presence of serum in 96-well plates. Growth in these follicles was significantly greater than follicles cultured in groups in 24-well plates in the presence of serum (day 2, $p=0.0041$; day 4, $p=0.0002$). Growth of these follicles was also highly significantly greater on both days of culture than the two control groups. Follicles cultured in the presence of serum in 24 well plates grew significantly more than control follicles in groups (day 2, $p=0.0079$; day 4; $p=0.0062$). Growth of group cultured serum-treated follicles was not different to follicles cultured individually in control medium (day 2 $p=0.1197$; day 4 $p=0.9701$). Follicles cultured in control medium in groups of three in 24-well plates grew significantly less than all other treatment groups on all days of culture. There was significant deterioration of 24-well control follicles, and many could not be measured by day two of culture, and so were discounted completely from the study from day 0. These results are illustrated in figure 4.3.1.

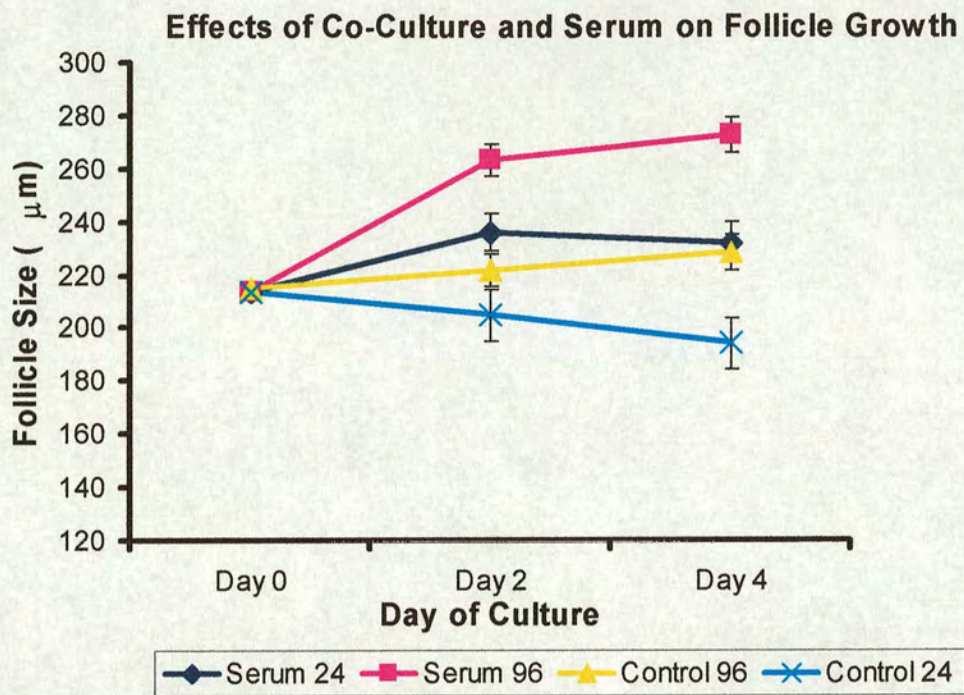


Figure 4.3.1: Effects of Co-Culture and Serum on Follicle Growth

Follicle growth was found to be significantly greater in follicles treated with serum and grown individually (serum-96; n=63; pink) than serum-treated group cultured follicles (serum-24; dark blue, n=66). There was no difference in growth in serum cultured follicles in groups in 24-well plates than control follicles in 96-well plates (control-96; yellow; n=54). Control follicles in 24-well plates grew significantly less than all groups on both days of culture (control-24; light blue; n=31). Values are mean \pm SEM.

4.3.2 Histological Examination of Cultured Follicles

Histological examination of cultured follicles revealed that follicles cultured in the presence of serum and cultured individually in 96-well plates displayed less atresia after four days of culture than all other treatment groups. There were significantly less follicles with >5% pycnotic granulosa cells in these follicles than all other treatment groups (compared to: Serum-24 $p=0.0148$; Control-96 $p=0.00016$; Control-24 $p=0.000$) {figure 4.3.2; table 4.3.1}. There was no significant difference between control follicles cultured in groups and those cultured individually ($p=0.074$). There were significantly more atretic follicles in group cultured control follicles than those cultured in groups in the presence of serum ($p=0.0045$). Oocytes were found to be significantly larger in follicles cultured in the presence of serum (figure 4.3.3; table 4.3.1) in comparison to controls. There was no significant difference in oocyte size when follicles were cultured in serum when cultured in groups or individually ($p=0.7$). There was also no difference between control follicles in terms of oocyte size when cultured in groups or individually ($p=0.85$). However, serum treated-follicles contained significantly larger oocytes compared to follicles cultured in its absence. There was however, significantly less oocyte degeneration in control 96-well follicles than those cultured in groups in control medium in 24-well plates (table 4.3.1). This difference was not significantly different in comparison to follicles cultured individually or in groups with serum supplementation. There were also significantly fewer follicles retaining an intact BM in follicles cultured in groups in 24-well plates in the absence of serum compared to follicles cultured in serum (table 4.3.1).

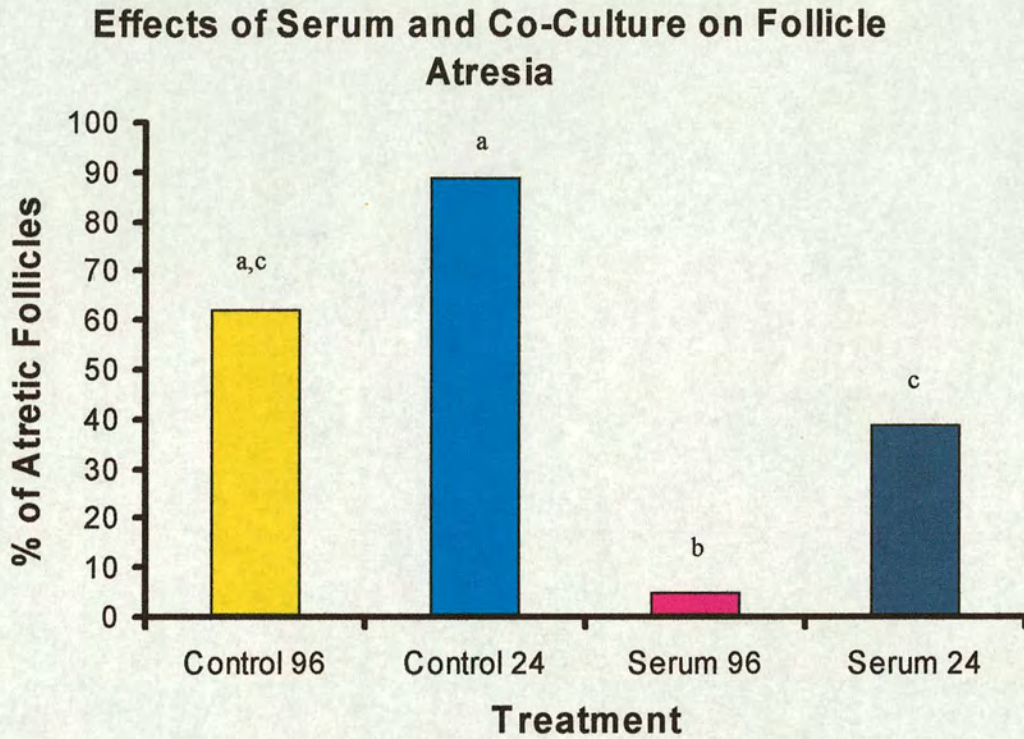


Figure 4.3.2: Effects of Serum and Co-Culture on Follicle Atresia.

Follicles cultured in serum in 96-well plates which were cultured individually had significantly less atretic follicles than any other treatment group. (Control 96 well; yellow; n=21); (Control 24 well; light blue; n=18); (Serum 96 well; pink; n=21); (serum 24 well, navy blue; n=18). Values are percentage of atretic follicles (>5% pycnotic granulosa cells) per treatment group. Different letter denotes significant difference in raw data.

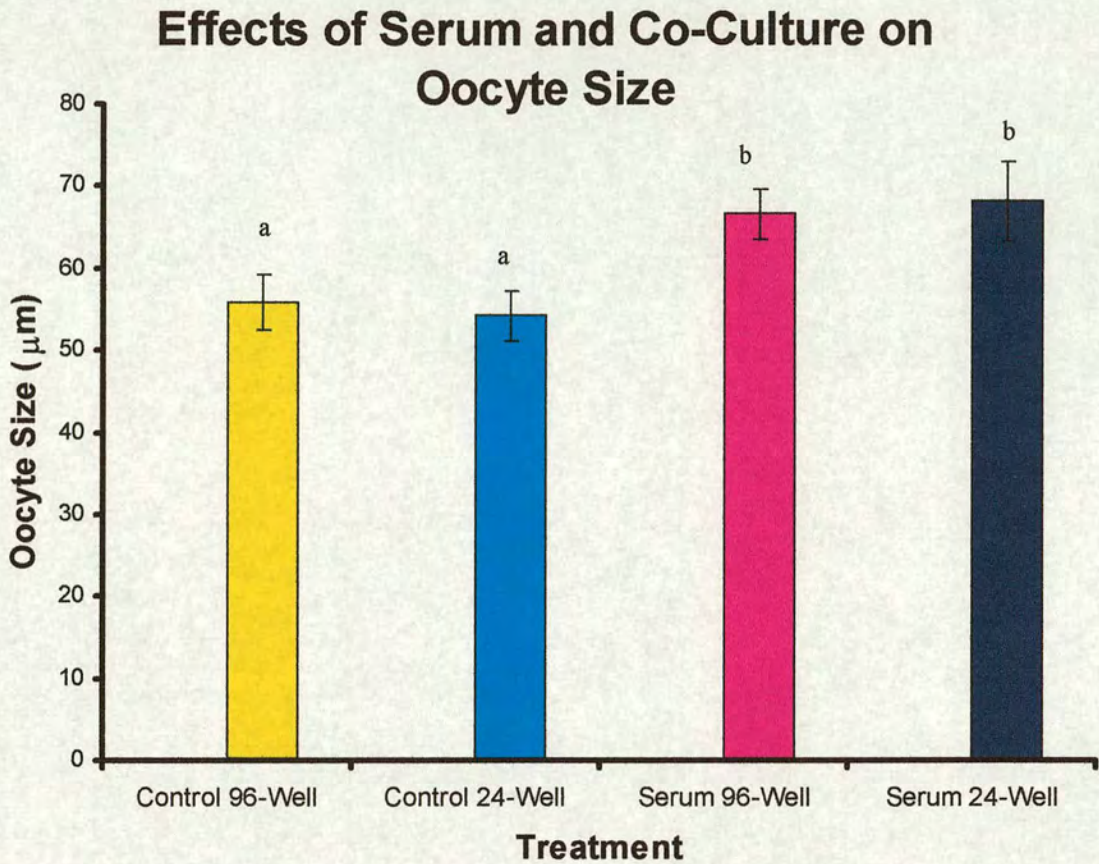


Figure 4.3.3: Effects of Serum and Co-Culture on Oocyte Size. Oocytes grown in serum had significantly larger oocytes than grown in the absence of serum. The mean oocyte diameters were not significantly different between control 96-well oocytes and control 24-well oocytes, but were both significantly smaller than serum-treated follicles. Serum 24-well oocytes and 96-well oocytes were not significantly different. (Control 96 well, yellow, n=21; Control 24 well, light blue, n=18; Serum 96 well, pink, n=21; serum 24 well, navy blue, n=18). Values are mean \pm SEM. Different letter denotes significant difference ($p < 0.05$).

Table 4.3.1: The Effects of co-culture and serum on follicle histology. The effects of co-culture, serum and different well types on oocyte diameter, number of theca layers, oocyte degeneration, granulosa cell pycnosis (atresia), theca degeneration, antrum formation, and basement membrane integrity are displayed in table 4.3.1.

Treatment	Mean	Mean No.	Oocyte	% Follicles	Theca	Antrum	Basement
	Oocyte	Theca	Degeneration	>5% GC	Degeneration	Formation	Membrane
	Diameter	Layers	(%)	Pycnosis	(%)	(%)	Intact (%)
	($\mu\text{m}\pm\text{SEM}$)*	($\pm\text{SEM}$)					
Control 96-Well (n=21)	55.9 ^a	2.05	66.6 ^{a,b}	61.9	19	4.7	61.9 ^{a,b}
	± 3.3	± 0.33					
Control 24 – Well (n=18)	54.2 ^a	2.2	77.8 ^b	88.9	22.2	11.1	44.4 ^a
	± 3.1	± 0.33					
Serum 96-Well (n=21)	66.6 ^b	1.28	38.1 ^a	4.8	28.6	28.5	85.7 ^b
	± 3	± 0.28					
Serum 24-Well (n=18)	68.2 ^b	1.17	38.9 ^a	38.9	38.9	22.2	66.7 ^{a,b}
	± 4.8	± 0.26					

* ±SEM: ±Standard error of the mean. Different letter denotes significant difference between groups (p<0.05). GC=Granulosa Cells.

4.4 Discussion

The results from this study indicate in all respects that follicles grew and survived best when cultured individually in 96-well plates in the presence of FSH, ascorbate, and serum. Follicle growth rates were greater when cultured individually in 96-well plates, and granulosa cell atresia was also reduced. However, the results from this experiment did not emulate those produced by Wu et al (2001). It is possible this could have been due to the medium in each well being increased to 500 μ l, compared to 280 μ l in the Wu et al study (2001), diluting any beneficial stimulatory factors produced by follicles. Possible problems affecting the viability of the present study could also have come from replacing half of the medium every two days. This means that any supplement which had been added to medium at the beginning of culture may have been used up by the follicles over two days in culture. This would mean that follicles would receive only half of the supplements when the medium was changed from day 2. For more accurate results it may be useful to replace the entire medium, in future experiments.

Probably the most successful culture system published for the development of porcine preantral follicles was by Hirao et al (1994), and similar results were obtained by Telfer et al (2000) using a similar system. A system was described whereby follicles were grown over a period of sixteen days, using a collagen matrix. Follicles were isolated using collagenase to dissolve the basement membrane, and were then cultured in the presence of serum in a collagen matrix. Of the surviving oocytes, around 20% were larger than 100 μ m. Of these oocytes, 60% which were $\geq 110\mu$ m were found to be capable of meiosis resumption. No

oocyte of $<90\mu\text{m}$ was capable of resuming meiosis. The starting size of oocytes in these follicles was $70\text{--}89.5\mu\text{m}$. Although these oocytes were matured *in vitro*, sperm penetration was observed only in a small number of oocytes (3/36), and emission of the second polar body was not observed in any of these fertilised oocytes. This probably indicates deficient cytoplasmic maturation of the oocytes, and something was lacking in the culture system that they were unable to complete the second polar body extrusion. The Wu group (2001) used oocytes of a similar starting size to the Hirao group, but found that oocytes could be grown to a stage where the oocytes could be matured, fertilised, and reach a blastocyst formation rate of 43%. This is a phenomenal rate of maturation for oocytes which began a four day culture in follicles at a size of $\sim 200\mu\text{m}$. Most of oocyte growth takes place when the follicle is in the preantral stages of development. To obtain fertilisable oocytes from preantral follicles after only four days of culture probably indicates the oocytes will be deficient in some way, as they have not had the time required to grow and develop in the follicular unit. The supplements used in the Wu system were added at high concentrations (see appendix 3 for discussion and comparison of supplements used in chapters 3 and 4), and this may have pushed development precociously to result in fertilisable oocytes after a very short culture period. The final size of follicles in the Wu experiment was found to be 48% larger on day 4 than day 0. In comparison this study found follicles were 22% larger when cultured in the absence of other follicles with serum, FSH and ascorbate, and only 10% bigger when cultured under similar conditions to the Wu paper, i.e. in groups. In the study by Mao et al (2002) it was found that follicles were 26% larger when

cultured in groups in 24-well plates with serum, ascorbate and FSH, but the concentration of FSH used in that experiment was four times higher than the concentration used here, thus greater follicle growth would be expected. The pattern of follicle growth over four days was similar in all systems tested, as a sharp rise was observed in all serum cultured follicles over the initial two days of culture, which then decreased by day 4.

In the present experiment it was found that after four days in culture oocytes had grown to an average size of 66.5µm in diameter in follicles cultured individually in the presence of serum, ascorbate and FSH. Oocytes of this size are incapable of the resumption of meiosis. Oocytes cultured in control follicles treated without serum were found to be significantly smaller than those cultured in serum, and degeneration rates were also significantly lower in oocytes cultured in serum, therefore serum with ascorbate and FSH acts as a survival factor for oocytes in this system, although they were not large enough to be fertilisable (Hirao et al, 1994).

In the study published by Wu et al (2001) follicles were punctured on day 4 of culture, and the oocytes were removed for *in vitro* maturation. It was decided in this experiment to fix follicles for histological examination to ascertain not only oocyte health and size, but also to examine follicle health. Histological examination of follicles revealed that almost every aspect of follicle and oocyte health was best when they were cultured in the presence of serum, ascorbate and FSH individually in 96-well plates. Oocyte health and antrum formation were better in follicles cultured in serum individually, although these differences were not significant. The number of follicles which were morphologically considered to be atretic was also

significantly less in individually cultured serum follicles than all other treatment groups. Follicles cultured in serum individually survived and grew better than those grown in serum and cultured in groups, and individually grown control follicles also survived better than group controls. It is not clear whether this is an artefact of the culture system or due to the presence of other follicles having an inhibitory effect. Some follicles cultured in-groups of three in the 24-well plates were observed to stick together, and also to the bottom of the culture well.

To improve this system to yield optimum growth and development of follicles it would be useful to grow the follicles with the support of a collagen matrix. The results from this experiment indicate that culture in 24-well plates is not beneficial to follicle development. The follicles were unsupported and rolled around in the medium, sometimes sticking to each other. There was significantly more pycnosis in serum follicles cultured in these wells compared to 96-well cultured serum follicles, indicating that culture in these wells had a very negative effect on follicle development. Also the system described by Wu suggested they used only 280µl of medium, which was barely enough to cover the bottom of the well. For this study 500µl was used. The use of serum in a follicle culture system is far from ideal, as this introduces undefined elements into the culture medium. The addition of serum makes it difficult to attribute the effects of a specific factor to any aspect of follicle development. An ideal system would be serum-free, and any added factors could therefore be considered directly responsible for any observed effects in culture. NCSU 23 is probably not a good medium as without serum follicles die quickly, as could be observed in control follicles in this system, which displayed high levels of

somatic cell pycnosis after only four days of culture. In comparison follicles can be grown in Weymouth medium for up to 30 days in a serum-free environment, and survive, so this medium may be a better choice for serum-free *in vitro* development of porcine preantral follicles.

The results from the present experiment did not emulate those produced by Wu et al (2001). This may have been due to the different amount of medium used in the two studies. In the present study it was found that medium used at a volume of 280 μ l evaporated very quickly. However, any stimulatory factors produced by growing follicles would therefore have been at a higher concentration in the smaller volume of medium. This may have enhanced follicle development and explain the high blastocyst formation results described in the Wu et al (2001) experiment.

**Chapter 5: The Secretion of the Gelatinases MMP-2 and
MMP-9 by Porcine Follicles and Oocytes *In Vitro***

5.1 Introduction

The processes of follicle development, ovulation, and corpus luteum formation are all associated with profound tissue remodelling and extracellular matrix turnover, and these developmental processes must be carefully controlled *in vivo* (Curry and Osteen, 2001). A family of proteases that are known to control extracellular matrix remodelling are the Matrix Metalloproteinases (MMPs), and their endogenous inhibitors, Tissue Inhibitor of Metalloproteinases (TIMPs). The ovary undergoes tremendous restructuring and remodelling during folliculogenesis (Smith et al, 1999), and from studies in other species MMPs have been shown to be involved in these processes. MMPs have been implicated in the latter stages of follicle development (Puistola et al, 1986; Curry et al, 1992), ovulation (Tadakuma et al, 1993), and luteolysis (Endo et al, 1993). Their role in the development of early follicles has not yet been established, if indeed they are involved at this stage of development. The main aim of this experiment was to examine whether MMPs are produced by porcine oocytes and follicles *in vitro*, and identify their potential as non-invasive, secreted markers of development.

The sub-group of MMPs that have been linked to folliculogenesis are the gelatinases, due to their ability to degrade collagen, laminin, and fibronectin (Curry and Osteen, 2001), the main components of the follicle basement membrane (Greenwald and Roy, 1994). There are two members of the subfamily gelatinases; MMP-2 and MMP-9, and in their catalytic domain they contain a fibronectin-like sequence, which enables them to bind and cleave gelatin, as well as other components of the basement membrane (BM).

MMPs and TIMPs have been found in the ovaries of several species, including human (Curry et al, 1990), cow (Juengel et al, 1994), sheep (Russell et al, 1995) and pig (Smith et al, 1994). Most studies have concentrated on the role of the MMPs in the latter stages of reproduction; however, MMPs are produced by early bovine follicles, where they have been identified as markers of bovine follicle health (McCaffery et al, 2000). In this latter study bovine preantral follicles were cultured individually *in vitro*, and analysis of culture medium revealed that secretion of MMP-9 by cultured preantral follicles was associated with low apoptotic cell death in somatic cells.

The main aims of this experiment were therefore:

- (1) To examine the secretion pattern of MMP-2 and MMP-9 from cultured porcine preantral follicles.
- (2) To examine the potential of MMP-2 and MMP-9 as non-invasive secreted markers of preantral follicle and oocyte health and development.
- (3) To characterise the secretion patterns of MMPs by different follicle cell types.

5.2 Materials and Methods

5.2.1 Preantral Follicle Culture Medium

Preantral follicles of size 175-275 μ m were isolated from fresh porcine ovarian tissue, as described in section 2.1.1. Follicles were cultured in 96-well plates in McCoys serum-free medium (supplemented as described in chapter 2.1.2.1) and cultured for 6 days. Half of the medium was removed and replaced on days 2, 4 and 6 of culture and then stored at -20°C. The follicles used for this study were cultured over six days, and fixed for histological examination using Bouins solution. Fixed follicles were then processed for histological examination, and stained using haematoxylin and eosin. Follicle pycnosis was measured by determining the percentage of pycnotic somatic cells present compared to healthy cells, and follicles with more than 5% pycnotic granulosa cells were classified as atretic (section 2.1.3 for details).

5.2.2 Collection of Oocytes and Culture of Cumulus-Oocyte Complexes and Denuded Oocytes

Conditioned oocyte and cumulus-oocyte complex (COC) medium was made by culturing denuded oocytes or intact complexes. Oocytes were aspirated from follicles of ovaries collected from slaughtered prepubertal gilts using a syringe and needle (Merck Ltd, Lutterworth, UK). Follicular fluid was removed from follicles, containing somatic cells and the oocytes. Follicular fluid was then placed in a 15ml alpha tube, until required. The cells were left to settle by gravity for several minutes, and the supernatant was then removed from each tube and 3ml of

Leibovitz medium (GIBCO BRL, Life Technologies Ltd, Paisley, Renfrewshire) was then used to wash the pellet at the bottom of the tube, containing the oocytes and unwanted somatic cells. The supernatant was removed once more after allowing the cells to settle, and 3ml of Leibovitz medium was added. Oocytes were then removed from the medium using a dissecting microscope (Olympus, UK) with a finely drawn glass capillary tube and placed in a small petri dish until required. They were then either treated with 600IU/ml hyaluronidase (SIGMA chemicals, Poole, Dorset) to remove cumulus cells, and were incubated at 37°C, or collected as COC. Oocytes were then washed in McCoys (GIBCO BRL, Life Technologies Ltd, Paisley, Renfrewshire) serum-free medium and put in 96-well plates with 1 oocyte or cumulus-oocyte complex cultured/ μ l of medium. Stripped oocytes and complexes were then cultured for 24 hours, and medium was subsequently removed and stored at -20°C until required.

5.2.3 Zymography and Detection of MMPs in Follicle and Oocyte Conditioned Medium

Gelatinase activity in medium was detected by gelatin substrate zymography on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% gels; mini-gel apparatus; BioRad, Hemel Hempstead, UK) containing gelatin (1mg/ml) under non-reducing conditions. The SDS which is present in the gels causes activation of the latent form of the MMPs, and active forms which are bound to the inhibitors, thus allowing their detection (Riley et al, 1999). Gels were washed twice (2x15min, 2.5% (v/v) Triton-X100; 2x2min TBS x 10), then incubated at

37°C in digestion buffer 50mM Tris; 200mM NaCl; 5 mM CaCl₂, 1μM ZnCl₂; 0.02% (v/v) Brij-35) overnight. Gels were then stained for 3 hours at 23°C with 0.5% Coomassie blue R250 in 30% methanol/10% glacial acetic acid in H₂O. Gels were then destained using staining solution without the Coomassie blue. Areas of the gel that contained clear patches indicated the presence of gelatinase activity, where the gelatin substrate had been hydrolysed by MMP gelatinase action. MMP-2 and MMP-9 were identified by comparison to molecular weight markers and control standards of human amniotic fluid collected during labour (Riley et al, 1999).

5.2.4 Data and Statistical Analysis

The activity of MMP-2 and MMP-9 was measured using densitometric analysis. Relative densities indicating activity (expressed as arbitrary absorbance readings) were derived from zymograms and compared with parallel background readings of equal area, and calculated using dedicated software (Quantity One; Bio-Rad). Readings were compared only with another gel run at the same time under exactly the same conditions (identical stains, buffers and incubation periods). Densitometric values indicating MMP activity were compared using Student's *t* test, and were only used to compare oocyte and cumulus-oocyte complex readings (Riley et al, 2001). Zymogram readings were not compared between treatments and production of MMPs as the gels were run on different days. The production of MMP-2 and -9 and correlations between treatment group and follicle atresia was compared using the Fisher's Exact Test.

5.3 Results

5.3.1 Detection of MMP-2 and MMP-9 in Preantral Follicle Conditioned Medium

In follicle conditioned medium from cultured porcine follicles it was found that the latent form of MMP-2 was the predominant gelatinase produced. All follicles cultured in ascorbate and FSH individually were found to produce this 72kDa protein, visualised by zymography. Detection of the latent form of MMP-2 was seen in almost all follicles in all treatment groups (figure 5.3.1.1). Detection of active MMP-2 (66kDa) was observed in some follicles. However, there was no consistent pattern of secretion of MMP-2 with the various histological parameters, such as somatic cell health and oocyte health. The secretion of latent MMP-9 was also observed in follicle conditioned medium, as a band of 92kDa. Follicles from all culture groups were found to secrete MMP-9 (figure 5.3.1.2), but not in all follicles, and secretion was not consistent with treatment or morphological criteria.

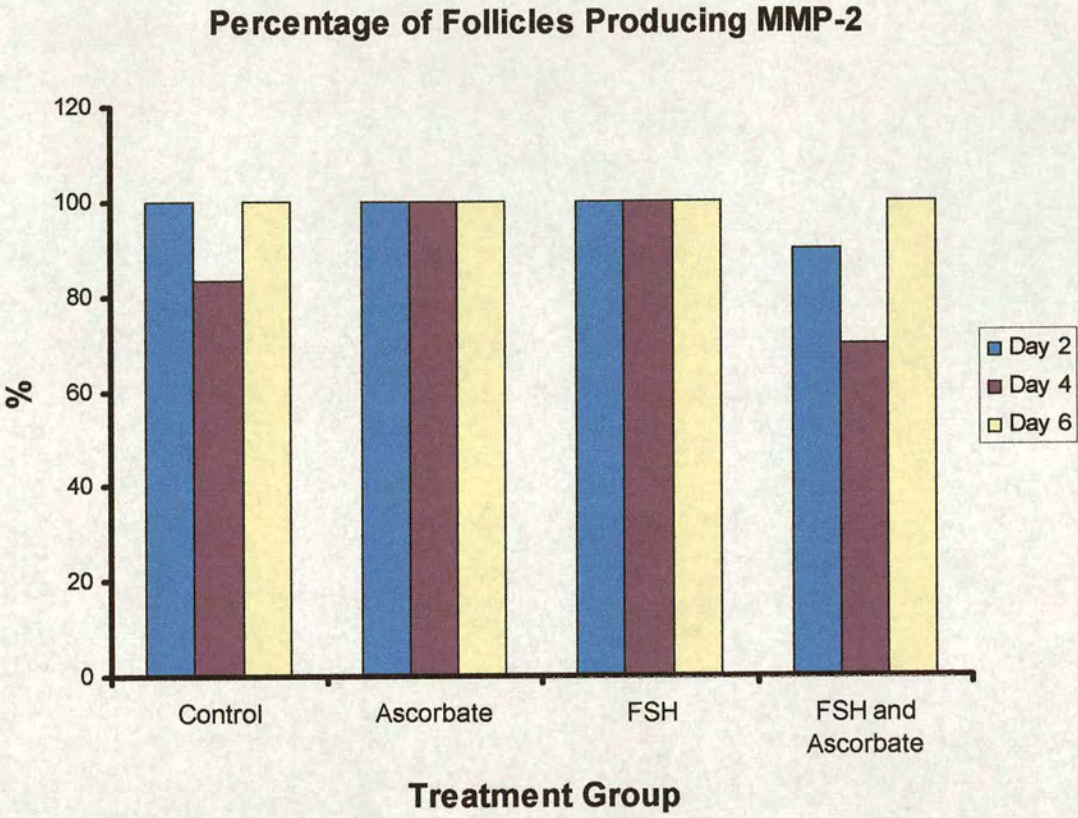


Figure 5.3.1.1: Percentage of Follicles Producing MMP-2. Production of latent MMP-2 by control (n=7), ascorbate (n=7), FSH (n=10), and FSH and ascorbate (n=10) treated follicles, expressed as a percentage of follicles secreting the gelatinase on days 2, 4 and 6 of culture.

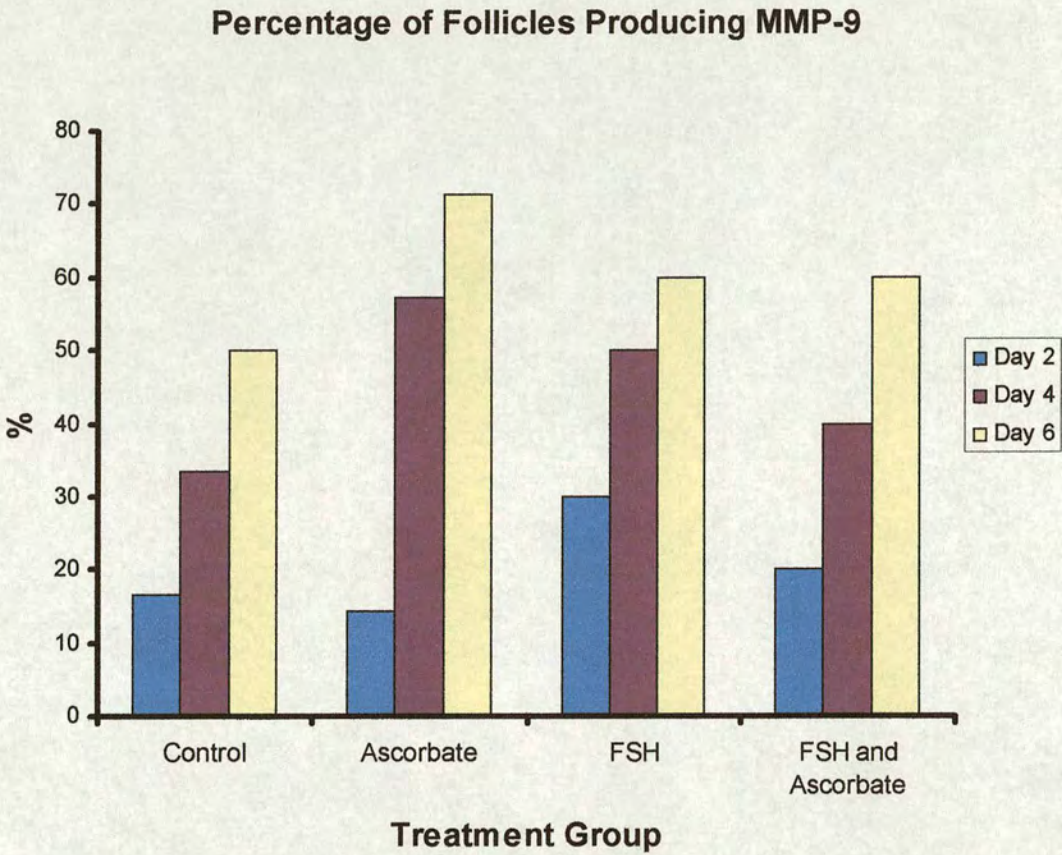


Figure 5.3.1.2: Percentage of Follicles Producing MMP-9. Production of latent MMP-9 by control (n=7), ascorbate (n=7), FSH (n=10), and FSH and ascorbate (n=10) treated follicles, expressed as a percentage of follicles secreting the gelatinase on days 2, 4 and 6 of culture.

5.3.2 Production of MMP-2 and MMP-9 by Cultured Follicles

The production of MMP-2 and MMP-9 was not found to be different between treatment groups. There was no pattern of secretion observed in any of the treatment groups. This is a compilation of several gels, giving a representation of the appearance of discrete bands indicating activity of the gelatinases MMP-2 and MMP-9, displayed in figure 5.3.2.

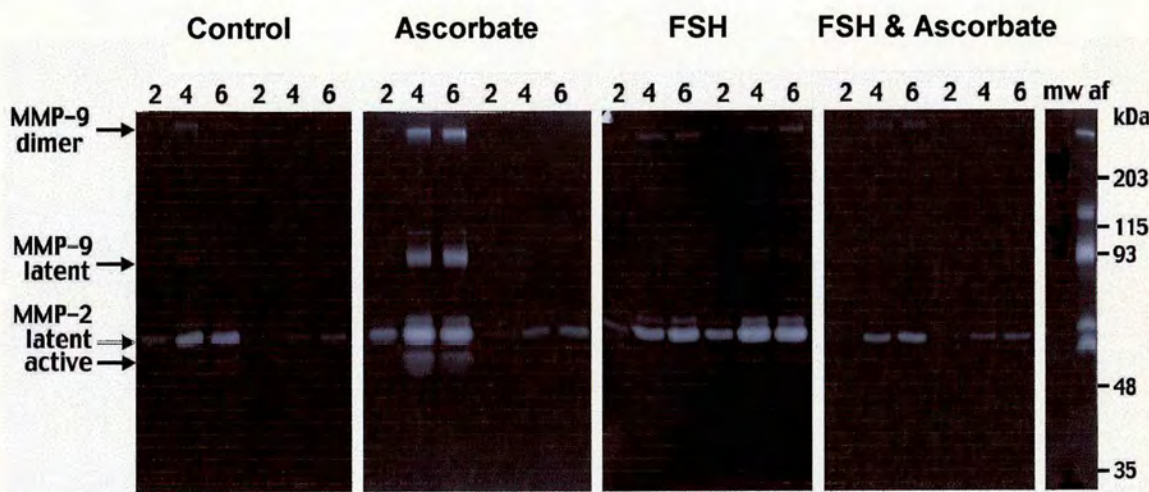


Figure 5.3.2 Zymogram of Production of MMP-2 and MMP-9 by Cultured Follicles. MMP-2 and MMP-9 production by cultured follicles is displayed on days 2, 4 and 6 of culture, when cultured in control medium, ascorbate supplemented medium, FSH medium, and FSH and ascorbate. (mw=molecular weight marker; af=human amniotic fluid).

5.3.3 Follicle Atresia and MMP-9 Production

It was found that there was no difference between production of MMP-2 and MMP-9 between follicles displaying varying degrees of somatic cell death. Pycnosis was reduced in follicles cultured in ascorbate, but treatment effects were not observed on the production of MMP-2 and MMP-9. There was slightly more MMP-9 production in follicles which displayed more than 5% pycnotic granulosa cells (i.e. atretic follicles), but this difference was not significant (figure 5.3.3, $p=0.16$).

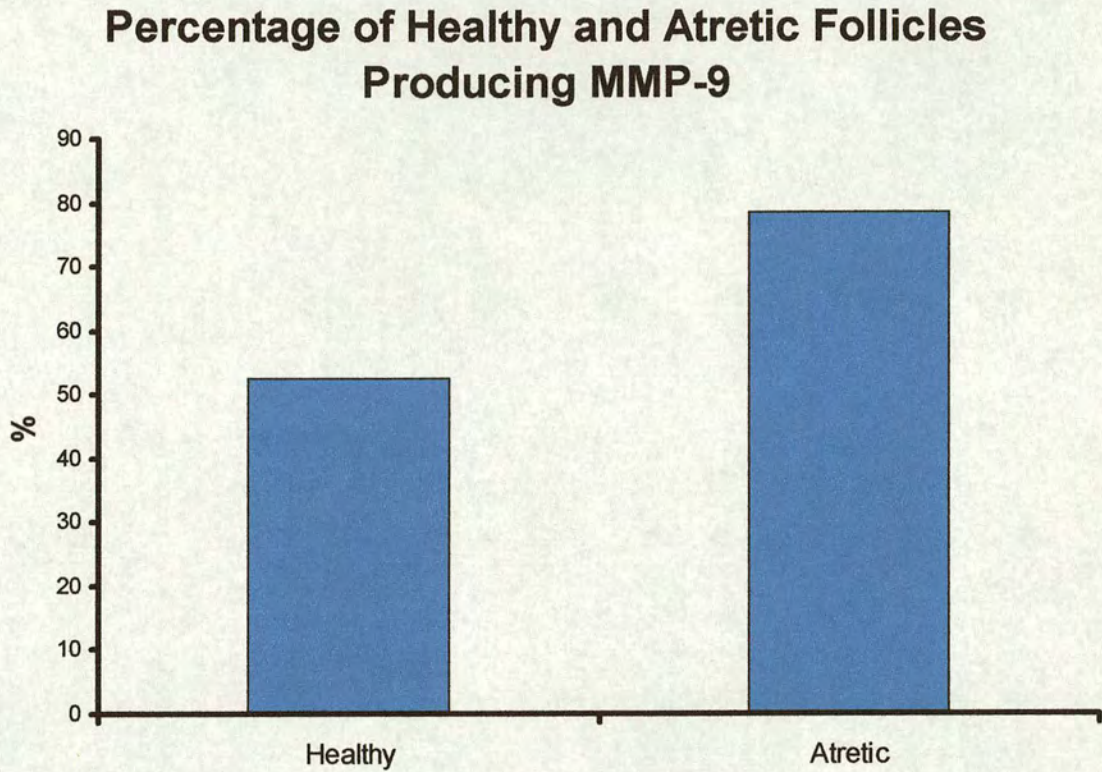


Figure 5.3.3: Percentage of Healthy and Atretic Follicles Producing MMP-9. The percentage of follicles displaying <5% pycnotic granulosa cells (healthy) which produced MMP-9 was 52.6% (n=19), in comparison to atretic follicles displaying >5% pycnosis, in which 78.7% of follicles were found to produce MMP-9 (n=14). There was found to be no significant difference (p=0.16).

5.3.4 Oocyte and COC Production of MMP-2 and MMP-9

It was found that the production of MMP-2 was much lower than in medium conditioned with denuded oocytes than in medium conditioned with cumulus-oocyte complexes. The bands for the latent form of MMP-2 observed were more intense when the complexes were cultured in comparison to denuded oocytes, but very little latent MMP-9 was observed in the resulting zymogram (figure 5.3.4). In contrast, stripped oocytes produced latent MMP-9, but very little MMP-2. Cumulus-oocyte complexes (coc) produced significantly more MMP-2 than denuded oocytes ($p=0.0003$), as displayed in figure 5.3.5.

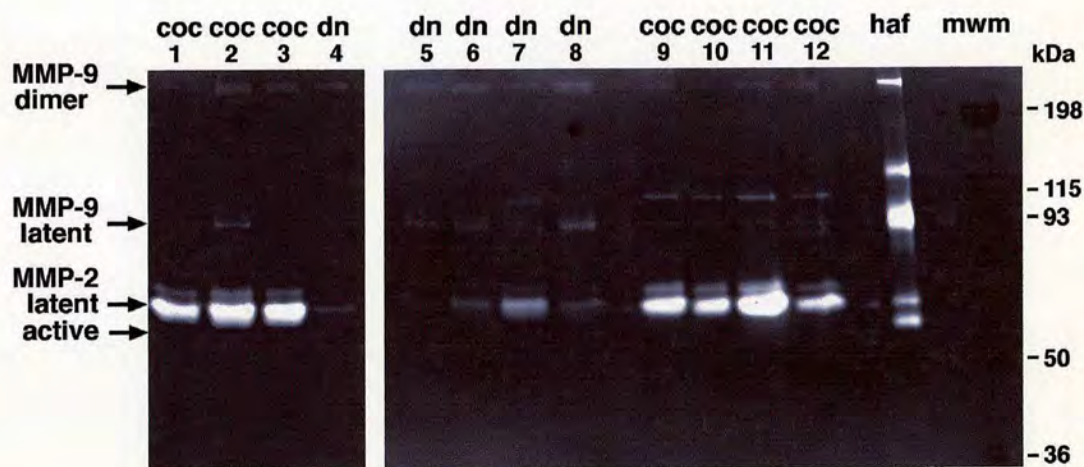


Figure 5.3.4: Zymogram of Production of MMP-2 and MMP-9 by Cultured Oocytes and Cumulus-Oocyte Complexes. MMP-2 production can be observed strongly from cultured complexes, but there was significantly less production of this factor from denuded oocytes ($p=0.0003$). In contrast, MMP-9 was produced more frequently when denuded oocytes were cultured without somatic cells, in comparison to oocytes cultured with cumulus cells. Control lanes contain human amniotic fluid (haf) and molecular weight marker (mwm).

**Latent MMP-2 Densitometry Readings of
Denuded Oocytes and COCs**

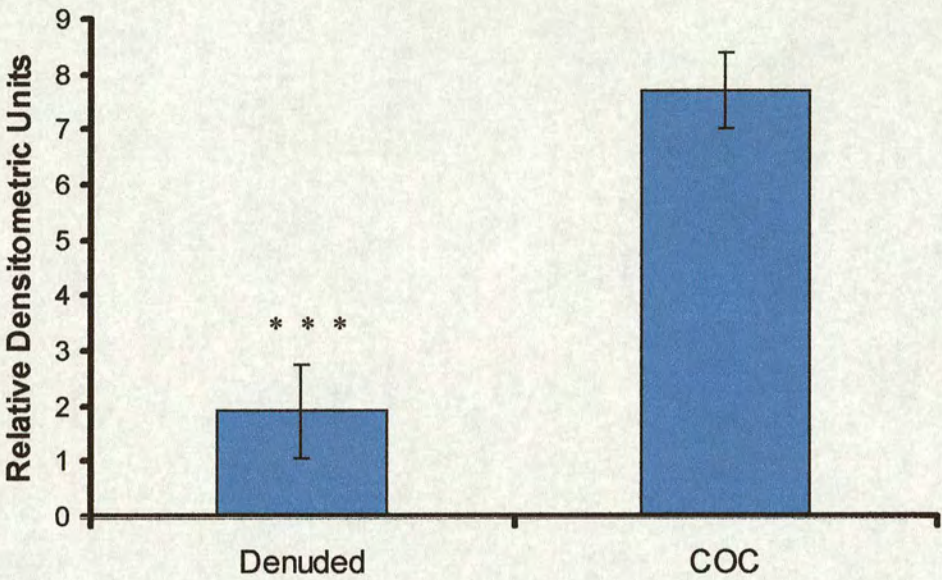


Figure 5.3.5: Latent MMP-2 Densitometry Readings of Denuded Oocytes and COCs. Relative abundance (arbitrary absorbance readings; mean ± SEM) of matrix metalloproteinase-2 (MMP-2) as determined by densitometric analysis. MMP-2 activity was significantly higher in cumulus-oocyte complexes {coc (n=7)} than in denuded oocytes (n=5) (p=0.0003) ***.

5.4 Discussion

The present experiment demonstrates that MMP-2 and MMP-9 are both produced by porcine preantral follicles when cultured *in vitro*. Preantral follicles cultured for six days in different treatment groups secreted MMP-2 and -9 on all days of culture. The most abundant gelatinase activity was the latent form of MMP-2 (72kDa), which was secreted by all follicles on at least one day of culture. Activity for MMP-9 (92kDa) was also detected, though less frequently than MMP-2. No obvious treatment effect on the secretion of the gelatinases was observed; it appeared to be a random event. There was also no apparent correlation between the secretion of MMP-2 and -9 and follicle viability.

The present study also demonstrates the secretion of MMP-2 and MMP-9 by porcine oocytes and cumulus-oocyte complexes. The predominant gelatinase secreted from the complexes was MMP-2. There was significantly more MMP-2 secreted from the complexes than the denuded oocytes. In contrast MMP-9 appears to be the predominant gelatinase secreted by denuded oocytes, and was secreted less frequently by cumulus-oocyte complexes. This could have been because MMP-9 is an oocyte-secreted factor, or it could be due to a suppressive effect on the oocyte of production of MMP-9 from the cumulus cells, due to some paracrine interaction. This may also be an indication that MMP-9 production from the oocytes may not be a sign of a health, as at the stage of development these oocytes should be attached to the cumulus cells. It may therefore be a sign of cell death or precocious development in the oocyte. It therefore seems likely that MMP-9 is an oocyte-secreted factor, and MMP-2 is secreted from the granulosa cells of the follicle.

Although the results suggest that MMP-2 may be a cumulus-secreted factor, further experiments would be required to confirm this, involving the culture of cumulus cells in the complete absence of oocytes. This would give a more accurate reflection of whether MMP-2 is indeed a cumulus produced factor, or if it is a product of cumulus-oocyte interactions.

Several problems arose in this study, which mean clear conclusions cannot necessarily be drawn from the results. Follicle viability was not linked to the production of the MMPs, as has been found in the cow (McCaffery et al, 2000). This could primarily be due to small sample numbers. If the experiment could be repeated using larger sample numbers a more accurate picture of the link between certain parameters of follicle and oocyte health could be established. However, even if the experiment were to be repeated with larger sample numbers, analysis of zymography is limited as gels can only be compared using densitometry if they are run on the same day. This is because variation occurs between gels run on different days, as variables such as running buffer and temperature can produce different results. For this reason numbers are limited to the number of samples which can be run in one day. One way to improve this for future experiments, and perhaps provide more conclusive results of a correlation between histological parameters or treatment effects and MMP production, would be to compare results on one day of culture only. It may also help to have less treatment groups. For example if ascorbate and control groups were compared only on day six, an effect may be observed due to larger sample numbers, as has been observed in the mouse and cow (mouse: Murray et al, 2001; cow: McCaffery et al, 2000).

The results from this experiment show that porcine preantral follicles produce both MMP-2 and MMP-9 *in vitro*. It was observed that the production of MMP-2 was seen in almost 100% of cultured follicles. Its production was far more abundant than MMP-9. This is consistent with findings in the horse and cow (McCaffery et al, 2000; Riley et al, 2001).

In the equine ovary it has been found that MMP-2 and MMP-9 were present in the follicular fluid of large antral horse follicles (Riley et al, 2001). The latent form of MMP-2 was found to be present in similar amounts in follicular fluid from follicles ranging from 10mm to 45mm in diameter. However, in these large follicles latent MMP-2 was present in all samples of follicular fluid, and was present in similar amounts in all follicle developmental stages tested (Riley et al, 2001). Some follicles also produced the active form of MMP-2, but there was no correlation between follicle size and this production. MMP-2 was also the predominant gelatinase secreted by cultured bovine follicles *in vitro*. A study by McCaffery et al (2000) looked at the secretion pattern of MMP-2 and MMP-9 in cultured bovine preantral follicles. MMP-2 was found to be the main gelatinase produced, but MMP-9 was also produced. MMP-9 production was directly correlated to follicle health. Follicles which displayed low levels of granulosa cell death were shown to be more likely to secrete MMP-9, thus identifying MMP-9 as a marker of bovine preantral follicle health. No such correlation was observed in the system described however, and the production of MMP-9 appeared to be inconsistent. There was slightly more MMP-9 produced by these follicles, which displayed >5% pycnotic granulosa cells, but this difference was not significant.

The production of MMP-9 in this follicle culture system appeared to be more sporadic. MMP-9 was not produced by all follicles, as was the case with MMP-2, which was produced by all follicles on at least one day of culture. MMP-9 was produced in the same frequency in follicles with pycnotic and healthy somatic cells, in follicles with degenerate and healthy oocytes, and in follicles that had or had not developed an antrum *in vitro*. There also appeared to be no treatment effect on whether or not MMP-9 was produced *in vitro*. It was found that there were no differences in the production of MMP-9 between treatment groups on any day of culture.

In all treatment groups an increase in the presence of MMP-9 was observed from day 2 to day 6. This increase could have been due to a cumulative effect, however, rather than an increase in production towards the latter stages of culture, as only half of the medium was removed on days 2 and 4 of culture. However, the increase in the proportion of follicles producing MMP-9 over a given culture period could possibly have increased due to precocious differentiation of the granulosa cells in culture. If they were developing at an accelerated rate due to culture conditions, they may have been producing MMP-9 too early. It could also be possible that the increased production of MMP-9 was an indication that the follicles were beginning to degenerate. It has been suggested that MMP-9 production by cultured bovine preantral follicles is indicative of follicle health; however the production of MMP-9 by antral bovine follicles has been found to be indicative of follicle degeneration (Khandoker et al, 2001). It is possible that the production of MMP-9 could be an indication of degeneration in porcine preantral follicles.

In the present experiment there appeared to be a slight increase in the number of follicles which produced MMP-9 when cultured in the presence of ascorbate and FSH compared to control follicles, both in combination and when ascorbate was added alone. Although this difference was not found to be significant, perhaps a greater sample number would have shown that it was. This is consistent with findings in the bovine, where it has been shown that addition of ascorbate to medium significantly increases MMP-9 production in comparison to controls (McCaffery et al, 2000). Murray et al (2001) also found that ascorbate increases the production of MMPs in mouse follicles *in vitro*. This could be an indication that the MMP system and ascorbate could be linked in their control of follicle remodelling and control of folliculogenesis. Densitometry of bands can be used to compare relative density of bands in different samples; unfortunately this comparison could not be made as samples were run on different gels, meaning quantification and comparison was not possible between samples, however a repeat of the experiment with more samples may show significant treatment differences.

The production of MMP-2 and MMP-9 in oocytes and COCs was also examined in this study, and it was found that oocytes which had been stripped of the surrounding somatic cells did not seem to produce MMP-9, in comparison to stripped oocytes, which appeared to produce the factor. In contrast MMP-2 was produced by COCs in relatively large quantities in comparison to stripped oocytes. The pattern of secretion revealed in the zymogram showed that COCs cultured in serum-free medium produced more MMP-2 compared to those cultured without the surrounding somatic cells, and this difference was found to be highly significant.

There was only one exception, where the denuded oocytes appeared to produce a little MMP-2 and did not produce MMP-9. It is possible this result was due to contamination with cumulus cells, as there was a denser band at 72kDa, corresponding to MMP-2. This result would imply that MMP-9 is in fact produced by the oocytes, and when the cumulus cells are present, the oocytes are somehow inhibited from producing latent MMP-9. This lower secretion of MMP-9 from the denuded oocytes is not due to the presence of TIMPs, as MMPs are still detected in samples whether they are bound to TIMPs or not, using zymography. Therefore if there were less MMPs present in the sample due to the presence of TIMPs, they would still be detected using zymography. This means that it is probably some kind of paracrine interaction between the cumulus cells and the oocyte inhibiting the production of MMP-9, rather than an inhibition by TIMPs. It also seems likely that MMP-2 is the major product of granulosa cells, and is therefore probably the principal gelatinase involved in follicle development. MMP-9 however, was produced less frequently by follicles cultured *in vitro*, and was not secreted by COCs, in comparison to denuded oocytes. It is possible therefore that this gelatinase is a principal regulator of oocyte development, and MMP-2 is critical for the development of follicles. Many oocyte factors have been identified in the past decade, and the oocyte has been shown to regulate its own, and also follicle, development in several ways, including cumulus expansion (Buccione et al, 1990; Vanderhyden et al, 1990), steroid receptor expression (Eppig et al, 1997), and follicle formation (Soyal et al, 2000) . It is possible that MMP-9 could also be an oocyte-secreted factor that could regulate follicle and oocyte development.

However, further studies are needed to show this. It is also possible that oocytes secrete different factors at different stages of development. Further studies would also examine whether oocyte secreted factors such as MMP-9 were secreted at specific stages of oocyte development, and identify whether they could be used as markers of developmental stage.

The production of MMPs and TIMPs has been studied in the latter stages of follicle development (Puistola et al, 1986; Curry et al, 1992). It was postulated that large amounts of protease activity would be required for the constant turnover and remodelling of the follicle, and for the ultimate rupture of the follicle, releasing the oocyte, and resulting in ovulation. This was found to be the case, especially with MMP-2, which has been found to increase in protein expression and activity up to ovulation (Puistola et al, 1986; Curry et al, 1992). Also, during the process of luteolysis, active forms of MMP-2 and MMP-9 are induced (Endo et al, 1993). In the rat ovary it has also been found that MMP-2 and MMP-9 expression increases as follicle development proceeds (Bagavandoss, 1998).

Although the role of the MMP system in late folliculogenesis, ovulation and corpus luteum formation and function has been studied, little has been done on the function of these gelatinases on early follicle development.

In the neo-natal rat ovary MMP-2 has also been found to be the most abundant gelatinase (Bagavandoss, 1998), in accordance with the results from this experiment, where MMP-2 was also found to be the predominant gelatinase secreted. The distribution of MMP-2 in the rat ovary was found to be abundant and wide-spread, as it was found in the granulosa, theca, interstitial cells and endothelial

cells of the PMSG-primed rat ovary using immunohistochemistry (Bagavandoss, 1998). Curry et al (1992) also found that MMP-2 was the main gelatinase in the rat ovary at the periovulatory period. MMP-2 therefore appears to be the principal gelatinase in follicular events before ovulation, and due to its wide-spread and abundant expression during folliculogenesis, it seems likely this gelatinase has a major role to play at all stages of follicle development.

In conclusion it has been shown that the gelatinases MMP-2 and MMP-9 are secreted by porcine preantral follicles when cultured *in vitro*. MMP-2 was found to be the predominant gelatinase secreted by follicles, as it was secreted by all follicles on at least one day of treatment. The production of MMP-9 by cultured follicles was a less frequent event.

The secretion of MMP-2 was also found to be significantly higher by cultured cumulus-oocyte complexes than cultured denuded oocytes. In contrast, MMP-9 appeared to be the predominant gelatinase secreted by the denuded oocytes compared to the cultured complexes, so it would appear that MMP-2 may be produced by the somatic cells of the follicle, and MMP-9 may be an oocyte-factor. For future experiments it would be useful to culture the theca cells of the follicle to examine their production of the MMPs, as this would provide a more accurate picture of the secretion patterns of the different follicle compartments. The use of larger sample numbers for the correlation of health of follicles, treatment effect and MMP production may also give a more accurate indication of the potential for MMPs as markers of oocyte and follicle developmental markers.

**Chapter 6: The Sequence and Expression of GDF-9 and
the Expression of BMP-15 in the Porcine Prepubertal
Ovary**

6.1 Introduction

Growth differentiation factor-9 (GDF-9) is a member of the transforming growth factor- β (TGF- β) family of growth factors, a family that plays an important role in the growth and differentiation of many different cell types involved in reproductive function (Shull and Doetschman, 1994; Elvin et al, 2000). GDF-9 was identified, along with GDF-3, using degenerate oligonucleotides corresponding to conserved regions of the TGF- β group among known family members (McPherron and Lee, 1993). Northern analysis revealed that GDF-3 transcripts were detected primarily in adult bone marrow, spleen, thymus, and adipose tissue; however, GDF-9 transcripts were detected only in the ovary. Further studies revealed that GDF-9 was not only ovary-specific, but was detected only in the oocytes of follicles (McGrath et al, 1995). In GDF-9 knock-out mice folliculogenesis is arrested at the primary stage of development, resulting in infertility (Dong et al, 1996).

It is well established that the somatic cells of the follicle influence the fate of the oocyte, and regulate its development by keeping it in meiotic arrest until ovulation (Pincus and Enzman, 1935), and providing it with requirements for growth and the eventual acquisition of meiotic competence (Eppig, 1991). This action was not thought to be reciprocated by the oocyte, until a body of work done in the 1990's showed that the oocyte can regulate its own development in several ways, including regulation of cumulus cell expansion (Buccione et al, 1990; Vanderhyden et al, 1990; 1992; Prochazka et al, 1991), steroidogenesis (Vanderhyden, 1993; Coskun et al, 1995; Vanderhyden & Tonary, 1995), and LH receptor expression (Eppig et al, 1997). Growth factors have also been shown to be produced by the oocyte, and regulate somatic cell function. One

such factor is GDF-9, and this factor has been shown to stimulate granulosa cell proliferation and increase the size of preantral follicles (Hayashi et al, 1999; Vitt et al, 2000).

The expression pattern of GDF-9 has been identified in several species, but not in the pig. Initial studies concentrated on rodent models and humans, and it was found that GDF-9 is localized exclusively in the oocyte (McGrath et al, 1995).

This study showed that GDF-9 was expressed only in the oocytes of follicles which had been initiated to grow, i.e. it was absent from primordial follicles.

Similar experiments using ovine and bovine material revealed a different expression pattern, as GDF-9 was found at all stages of follicle development, including primordial; however, it was still found only in the oocyte (Bodensteiner et al, 1999).

The question of whether GDF-9 is indeed completely oocyte-specific has been called into question. A study was carried out to reveal whether GDF-9 is oocyte-specific, and it was shown that GDF-9 may not be oocyte-specific

(Fitzpatrick et al, 1998). Northern analysis showed that in mouse tissue GDF-9 was present in the testis, the ovary, and most surprisingly the hypothalamus.

GDF-9 was also found in the pituitary, testis and uterus of the human, and in the ovary and testis of the rat. Previous studies of rodents and other species showed that GDF-9 was oocyte-specific (McGrath et al, 1995; Aaltonen et al, 1999;

Bodensteiner et al, 1999; Jaatinen et al, 1999). To my knowledge there has been no other study conducted which has found GDF-9 in any tissue other than in the ovary, and more specifically, the oocyte. This would bring into question

whether the result obtained by the Fitzpatrick group could possibly be the identification of unspecific targets in their PCR and Northern blots. The TGF- β

family are a large family of structurally related polypeptides (Massague, 1998), and it seems possible that their structural similarity could mean that they may be picked up as non-specific products of PCR and Northern blots, when using probes for the detection of GDF-9, as carried out by Fitzpatrick et al (1998). The research which was done by this group in 1998 has not yet been emulated to my knowledge, and this may suggest that false positives could have been identified in this research.

Bone morphogenetic protein-15 (BMP-15), also known as GDF-9 β , is a closely related TGF- β family member of GDF-9, and is thought to be oocyte-specific. It has been shown that BMP-15 regulates granulosa cell proliferation and differentiation, as it regulates granulosa cell mitosis, and kit ligand expression (Otsuka and Shimisaki, 2002). Mutations in BMP-15 are linked to both an increased ovulation rate in sheep, and can also cause infertility, in a dose dependent manner (Galloway et al, 2000). Thus it is apparent that BMP-15 plays a vital role in female reproductive function. BMP-15 expression has not yet been identified in the pig.

The main aims of this experiment were:

- (1) To identify the exon 2 portion of GDF-9 in the porcine ovary.
- (2) To identify similarities and differences in sequences between the pig and other species in the region of exon 2.
- (3) To examine the expression pattern of GDF-9 in the porcine ovary to establish firstly whether it is oocyte-specific.
- (4) To examine the expression pattern of BMP-15 in porcine follicular cells and determine its specificity.

6.2 Materials and Methods

6.2.1 RNA Extraction

RNA was extracted from porcine oocyte, granulosa and intestinal cells using the TRI REAGENT protocol (SIGMA, Missouri). Briefly tissue was homogenised using a polytron (System PT 1300D, Kinematica AG, Switzerland). An appropriate volume of tri reagent was added to the tissue samples (1ml for intestine samples and 0.5ml for oocytes and granulosa cells), and they were spun in a centrifuge for 10 minutes, at 12000 x g and 4°C. The clear supernatant was removed and added to a fresh tube. The sample was allowed to stand for 5 minutes at room temperature, and 200µl of chloroform was added/ml of tri reagent used. The sample was shaken vigorously for 15 seconds and allowed to stand for 5 minutes. The resulting mixture was then centrifuged at 12000 x g for 15 minutes at 4°C. The colourless aqueous phase containing ribonucleic acid (RNA) was removed and transferred to a fresh tube, and 500µl of isopropanol/ml of tri reagent was added. This mixture was centrifuged at 12000 x g for 10 minutes at 4°C. The RNA precipitate could then be observed at the side of the tube. The supernatant was removed, and the pellet washed using 75% ethanol. The sample was then vortexed, and centrifuged at 7500 x g for 5 minutes at 4°C. The ethanol was removed, and the pellet briefly air-dried. The sample was then re-suspended in diethyl pyrocarbonate (DEPC) water.

6.2.2 cDNA Construction

RNA was extracted from porcine oocyte, granulosa and intestinal cells using the TRI REAGENT protocol (SIGMA, Missouri). Complementary deoxyribonucleic acid (cDNA) was constructed from RNA obtained from

porcine oocytes using the advantage kit and protocol (BD Clontech, Basingstoke, UK). Primers were designed based on comparisons of the known sequences for human and mouse GDF-9. Primers were chosen which had been successful in the cow and sheep (Bodensteiner et al, 1999), and contained an approximate 50% composition of G/C base pairs (appropriate G/C content of between 45-60% is essential to give correct annealing temperature, and to give appropriate hybridisation stability). The primers chosen did span one exon, which is not ideal for primer design as ideally they should span an intron to avoid DNA contamination; however these primers were chosen due to success in ovine and bovine samples (Bodensteiner et al, 1999).

5'TAGTCAGCTGAAGTGGGACA3'

5'ACGACAGGTGCACTTTGTAG3'

RT-PCR was carried out using 224ng of total RNA. Reactions were run on the following programmes using a Techne Touchgene thermal cycler.

RT: 42°C for 1 hour

PCR:

94°C 2 min

94°C	}	30 sec	x35
40°C		30 sec	
72°C		1 min	

72°C 2 min

Products were then run on a 1% agarose gel with 0.5µg/ml ethidium bromide at 70V for 1 hour. Positive bands were identified under UV light, and excised from the gel using a sterile scalpel. PCR bands were placed in eppendorf tubes and weighed. DNA was then extracted from the agarose using a microcentrifuge and the QIAGEN QIAquick Gel Extraction kit (Qiagen Ltd, West Sussex, UK). On

the attainment of positive bands the reactions were repeated to obtain a large amount of product.

6.2.3 Cloning

DNA products of GDF-9 were used in ligation reactions using the PGEM-T easy vector as described in the PGEM-T and PGEM-T Easy Vector systems manual (Promega Ltd, Madison, USA). Transformations were also carried out as described in the manual. Plasmid mini preparations were carried out for positive results using the QIAGEN Mini Plasmid Prep kit (Qiagen Ltd, West Sussex, UK).

6.2.4 Localisation of GDF-9

RT-PCR was carried out on oocyte and granulosa cell RNA to identify the location of expression of GDF-9 in the porcine ovary. RT-PCR was carried out using Promega Access RT-PCR system (Promega, Madison, USA) using the following programme on the Techne touchgene cycler:

48°C	45min	
94°C	2 min	
94°C	30 sec	x40
60°C	1 min	
68°C	2 min	
68°C	7 min	

40 cycles of RT-PCR were carried out initially on 5ng of total RNA, and this was reduced to 25 cycles for GDF-9 samples. RT-PCR was carried out using the same primers as above for GDF-9, and BMP-15 sheep primers were also used

on the oocyte and granulosa cell RNA. RT-PCR was carried out using the same conditions as above with the following primers:

BMP-15 primers 5' AATCTCTCCTGCCATGTGG

3' GCAATGATCCAGTGATCCCA

6.2.5 In Situ Hybridisation and Localisation of GDF-9

6.2.5.1 Probe Construction

Probes for use in *in situ* hybridisation (ISH) were constructed for GDF-9 using a sense (sense probe negative control) and anti-sense (positive) orientated insert. Whole ovary sections were prepared and mounted on slides for ISH. The control slides were ovary sections with sense probe, and positive slides were prepared by adding anti-sense probe to whole ovary sections. The DNA was linearised by digestion with ECor1 (New England BioLabs, Herts, UK), and run on 0.7% agarose gel at 125V for two hours. Bands were then cut out under UV light using a sterilised scalpel, and placed in eppendorf tubes. DNA was then extracted using the QIAGEN QIAquick Gel Extraction kit. For the anti-sense reaction the probe was labelled using ³⁵S (Amersham Pharmacia Biotech, Buckinghamshire, UK) by adding 1µg of linearised template to 6µl of polymerase buffer (Promega, UK), 3µl 100nM DTT (Promega, UK; reducing agent for enzyme activity), 1µl RNAsin (Sigma, UK), 3µl 10x ³⁵S nucleotides to make RNA: CTP (5 µl of 100mM), GTP (5µl of 100mM), UTP (10µl of 1mM), ATP (5µl 100mM). 2µl of T7 polymerase (New England BioLabs, UK) were then added, followed by 4µl of 35S-UTP. For the sense reaction amounts were halved, and Sp6 was used instead of T7. The reaction was then incubated at 37°C for 1 hour. 2µl of T7, and 1µl Sp6 were then added to anti-sense and sense

reactions respectively, and incubated for 1 hour at 37°C. 2 μ l of DNase (Roche) were then added for 15 minutes at 37°C. The reaction was subsequently stopped with 2 μ l of ethyl-diamino-tetraacetic acid (EDTA, which acts as a calcium chelator), and the reaction products were stored overnight at -20°C. The following morning the counts per minute (cpm) were measured using a scintillation counter, and an appropriate volume was added to the slide to get 1x10⁶ cpm.

6.2.5.2 Slide preparation

Sections were de-waxed through 1x10 minutes in xylene, followed by 2x2 minute washes in 100% ethanol. Slides were then put through graded alcohols (90%, 70% and 30%) for two minutes each. Sections were then washed in sodium chloride (NaCl; 0.85%), followed by a 5 minute wash in phosphate buffered saline (PBS). Slides were then washed with Proteinase K (2 μ g/ml) in Tris and EDTA at pH 8, followed by a 5 minutes PBS wash, then a rinse in H₂O. The slides were then washed twice in NaCl / sodium citrate buffer (SSC) buffer, followed by a 10 minute wash in 0.1M triethalone (TEA) and acetic anhydride. The slides were then washed twice in SSC at 4°C for two minutes each wash. The probe was then added to each slide, and covered with a coverslip. They were then placed in a plastic bag, then a box, and stored overnight at 55°C.

6.2.5.3 In situ Hybridisation

Slides were soaked in 2xSSC washes at 55°C. The cover-slips were then carefully removed in beakers of SSC. The 2xSSC washes were then repeated 2x15 minutes at 55°C on a shaker. An RNase digestion was then carried out (20µg/ml in 2xSSC) at 37°C for one hour. The slides were then washed: 1x (2xSSC & 0.1% β-mercaptoethanol) at 55°C for 15 minutes; 1x (1xSSC & 0.1% β-mercaptoethanol) at 55° for 15 minutes; 1x (1 x SSC / 50% formamide & 0.1% β-mercaptoethanol) at 55°C for 30 minutes; 2x (0.1xSSC & 0.1% β-mercaptoethanol) at 55°C for 15 minutes. The slides were then dehydrated in 30, 70, 90, and 100% alcohol for two minutes each. They were then stored overnight in a plastic box with tissue soaked in ethanol.

6.2.5.4 Developing

In the dark room slides were dipped in developer for 2 minutes, followed by 1 minute in 1% acetic acid, and 4 minutes in sodium thiosulphate. Slides were then dipped in water. Sections were then stained using haematoxylin (2 minutes) and eosin (30 seconds). The slides were then rehydrated in graded alcohols, 70%, 90%, 2x100%, and then placed in xylene. Slides were then mounted using DPX and cover slips. Positive results showed up as silver grains against purple and pink of the haematoxylin and eosin. The slides were examined under the microscope.

6.3 Results

6.3.1 Sequencing of 277bp GDF-9 Fragment

Amplification of porcine cDNA with primers directed against homologous regions of human and mouse exon-2 produced a 277-bp fragment from porcine oocyte RNA. The fragment was verified by sequencing using Sp6 and T7 and an ABI 373XL sequencer. Nucleotide sequences of the fragment displayed 88% homology with the human sequence (figure 6.3.1). Sequences were compared using the BLAST 2 sequence comparison.



Figure 6.3.1 Comparison of Porcine and Human GDF-9 Exon 2 Sequences. Sequence of porcine GDF-9 exon 2 region in comparison to human GDF-9 exon 2.

6.3.2 GDF-9 Location by RT-PCR

Results from RT-PCR showed that GDF-9 was present in oocyte samples. A very faint band was observed for granulosa cells after the sample was run on a 1.2% gel at 100V after 40 cycles of PCR. However this band was not visible after 1 hour. No band was observed for granulosa cells and intestine cells after 25 cycles of PCR, but a band could still be observed from the oocyte sample. Every RNA sample used tested positive for GAPDH.

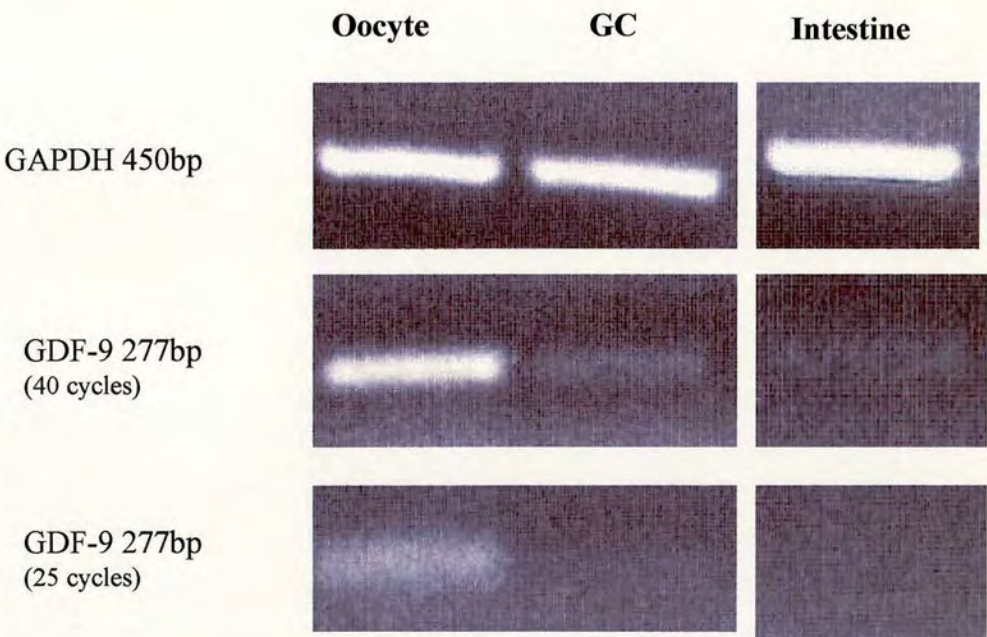


Figure 6.3.2 Expression of GDF-9 in Porcine Oocyte, Granulosa and Intestine Samples. Oocyte, intestine and granulosa cell samples after half an hour on a 1.2% gel after PCR of 40 cycles and 25 cycles using GDF-9 primers. GC=granulosa cells.

6.3.3 BMP-15 Location by RT-PCR

Results of the RT-PCR using 40 cycles showed a positive band for the oocyte sample, but no band in granulosa and intestine samples.

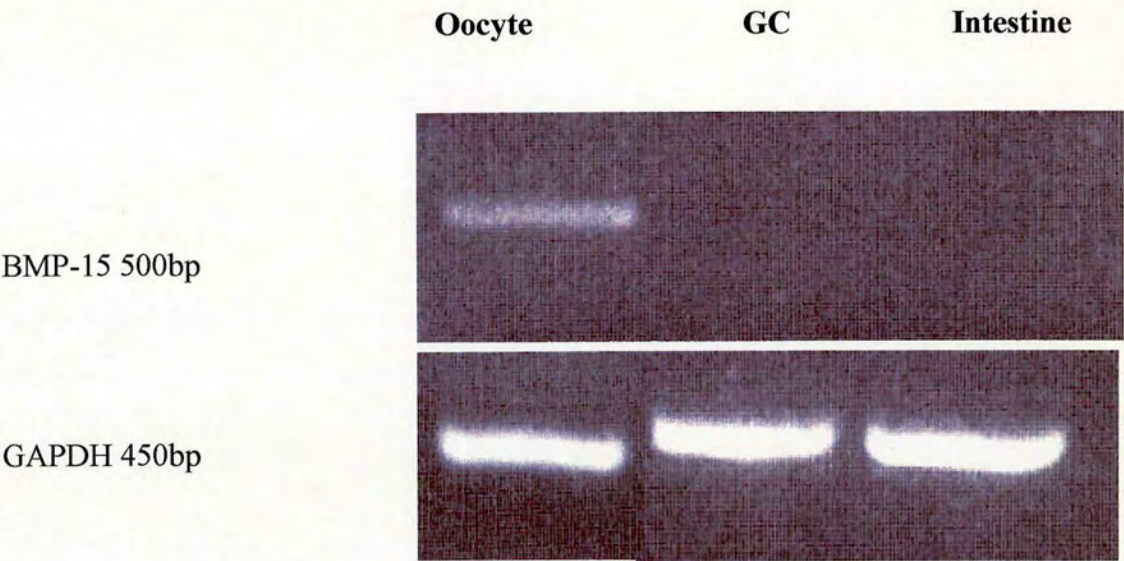


Figure 6.3.3 Expression of BMP-15 in Porcine Oocyte, Granulosa and Intestine Samples: Oocyte, intestine and granulosa cell samples after an hour on a 1.2% gel at 70V after 40 cycles of PCR. GC=granulosa cells.

6.3.4 In Situ Hybridisation

Results from *in situ* hybridisation did not show conclusive results as staining was not specific to any particular tissue type, and was also observed in the sense probe negative controls (whole ovary section with sense probe added). In this slide the oocyte appeared to have more grains than the rest of the tissue and the antrum.

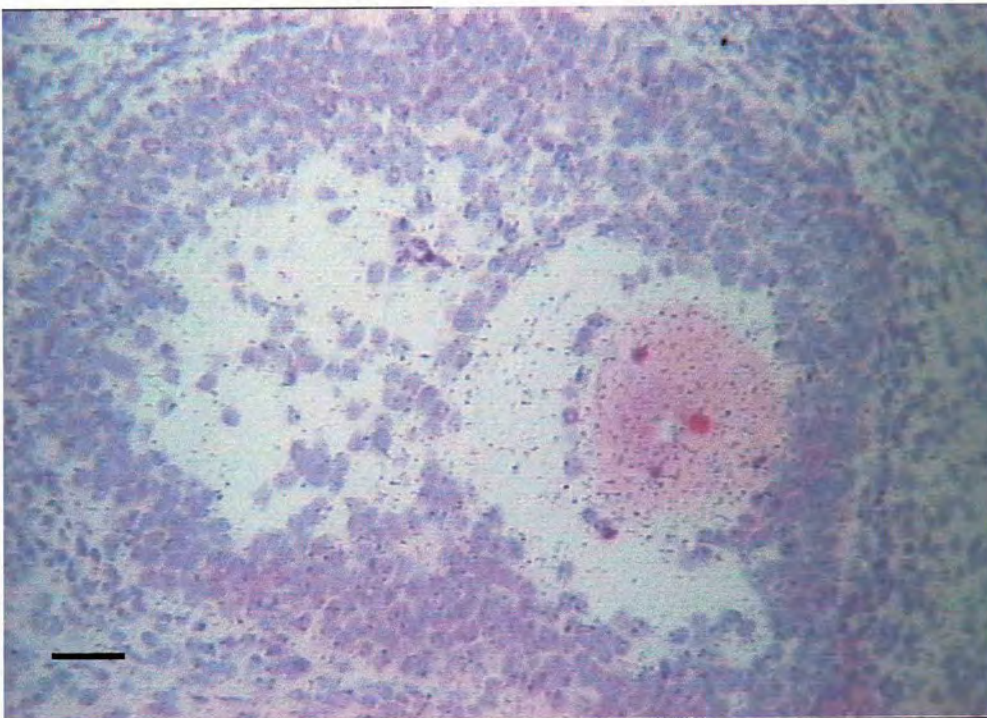


Figure 6.3.4 In Situ Hybridisation for GDF-9 in Porcine Antral Follicle. Antral follicle from porcine ovarian slice stained with ^{35}S grains. The oocyte appears to have slightly more grains than the somatic cells and at the antrum. Bar represents 30 μm .

6.4 Discussion

The results from this experiment indicate that porcine GDF-9 is probably an oocyte-specific factor, and it displays a high degree of homology to other species across a conserved portion of the gene. The 277bp fragment which was isolated in this experiment using primers based on the human and mouse exon 2 showed 88% homology to the human sequence and 84% to the mouse. The high homology which is displayed across the species in the mature region of the gene indicates the importance of this region, and its essential role in reproduction across the species. To complete this study, the whole region of GDF-9 would have to be identified in the porcine, which in the time-constraints of this PhD could not be achieved. The 277bp fragment which was isolated here is known to be a highly conserved region of the GDF-9 gene (Bodensteiner et al, 1999); however it is unlikely that this high degree of homology would be observed across the entire gene. The human, mouse and sheep GDF-9 gene spans around 2.5kb, and contains two exons and one intron. The homology of the sheep GDF-9 is 77% and 66% between human and mouse respectively (Bodensteiner et al, 1999). It therefore seems that the 277bp fragment is very highly conserved in comparison to the rest of the gene. The highly conserved nature of the 277bp fragment across the species indicates that this region is essential for reproduction, and this has stopped inter-species variation from occurring. The 277bp fragment of DNA has been conserved for millions of years by natural selection, as the last common ancestor shared by humans, pigs and mice was probably before the Palaeocene era, which began 65 million years ago (The Smithsonian Institution website). It was during this period that mammals evolved, and primates began to roam the earth. If the last common ancestor for

pigs, primates and mice was over 65 million years ago, this implies that strong natural selection must have taken place in order to conserve this section of the gene over such a long period of time, indicating its tremendous importance.

Results from the present study indicate that BMP-15 could also be an oocyte-specific factor, which is consistent with findings in other species (mouse: Dube et al, 1998; human: Aaltonen et al, 1999; rat: Jaatinen et al, 1999; sheep: Juengel et al, 2002). In the mouse BMP-15 is expressed in oocytes shortly after they enter the growing pool of follicles, and this continues until after ovulation (Dube et al, 1998). In this study oocyte RNA showed a clear positive result, whereas no result was obtained from granulosa or intestine RNA.

Initial expression analysis studies of GDF-9 in ovarian tissue showed that it appeared to be oocyte-specific (mice: McGrath et al, 1995; human: Aaltonen et al, 1999; rat: Jaatinen et al, 1999; sheep: Bodensteiner et al, 1999; Juengel et al, 2002; cow: Bodensteiner et al, 1999). This was disputed by Fitzpatrick et al (1998) where GDF-9 was discovered not only in the oocyte, but in several other tissue types other than ovarian in rodents and humans. In the present study initial RT-PCR experiments did not reveal the presence of GDF-9 in granulosa cells, but it was found in the oocyte. Subsequent RT-PCR was carried out using intestine as a negative control. PCR products were run out on a gel, and a very faint band was observed in both the granulosa and intestine samples, with a strong band observed in the oocyte sample. The bands from the granulosa and intestine samples were only visible after the gel had been running for half an hour, and disappeared after one hour. It would therefore seem likely that was not a positive result, and was more likely to be an artefact of the PCR. It could be that the weak positive bands observed after one hour of electrophoresis were

due to very sensitive PCR techniques and it may be that the gene is present at a very low level, but is probably not physiologically significant in these populations of cells. With a reduced number of PCR cycles it was also found that no band was present in the granulosa and intestine samples, but a positive result was still seen for oocyte samples. The results therefore indicate that GDF-9 is probably oocyte-specific in the pig. However, it could also be possible that as GDF-9 is a member of the TGF- β superfamily, which are structurally related polypeptides (Massague, 1998); it seems possible that the data obtained in the PCR and ISH experiments could be due to unspecific products produced due to the structural similarity of this family of proteins.

GDF-9 has been shown to be involved in the regulation of folliculogenesis in several ways. GDF-9 deficient mice are incapable of producing follicles beyond the early preantral stages, and are thus rendered infertile (Dong et al, 1996). The ovaries of mice homozygous for the mutation were found to have significantly smaller ovaries than wild-type mice, and although many primordial and early preantral follicles were present, no follicles beyond the one layer stage were observed. Further analysis of oocytes from mutant mice revealed that GDF-9 homozygous mutant mice oocytes grow more rapidly than control oocytes and that follicle growth ceases at the type 3b stage (Carabatsos et al, 1998). Mice deficient for the gene also produce oocytes which are abnormally large, and have structural defects, such as a lack of cortical granules. However these oocytes are capable of resuming meiosis, and have the ability to reach meiotic completion (Carabatsos et al, 1998).

GDF-9 has also been shown to regulate somatic cell development as *in vivo* application leads to an increase in the number of primary and preantral follicles,

and a decrease in the number of primordial follicles (Vitt et al, 2000). This implies GDF-9 plays a major role in folliculogenesis, and is vital for appropriate somatic cell differentiation.

BMP-15 is also essential for folliculogenesis. A natural mutation of the BMP-15 gene exists in sheep, and homozygotes for this mutation display impaired follicle production past the primary stage (Galloway et al, 2000). In contrast heterozygotes for the condition have an increased ovulation rate, and it has been postulated that mutants with an inconsistency in the receptor gene display increased ovulation rates due to a possible increase in SMAD phosphorylation (Souza et al, 2001). BMP-15 is oocyte-specific in humans, rodents and sheep (Aaltonen et al, 1999; Dube et al, 1998; Jaatinen et al, 1999; Juengel et al, 2002), and this is consistent with the preliminary results obtained from the present study.

In conclusion, results from the present study indicate that BMP-15 is probably oocyte specific in the pig, and GDF-9 appears to be oocyte-specific, but further studies are required to confirm these preliminary findings. GDF-9 and BMP-15 have both been shown to be essential for normal follicle development, and infertility results from a knock-out of these genes (Dong et al, 1996; Galloway et al, 2000). The identification of GDF-9 and BMP-15 in the pig using primers based on other species indicates the importance of these genes across the species, and their highly conserved nature.

Chapter 7: Discussion and Conclusions

7 General Discussion

The experiments described in this thesis were carried out to achieve two main aims; firstly to identify factors affecting early preantral follicle development using a serum-free *in vitro* culture system, and secondly to identify putative markers of ovarian follicular development. The experiments were carried out on pigs, as these animals are potentially a useful model system to mimic human follicle development, and pigs are also of potential importance for techniques such as cloning, xenotransplantation and *in vitro* fertilisation. Obviously the parallels which can be drawn between humans and pigs are limited, in terms of follicle development. The pig is a polyovular species, producing on average 10-20 offspring per successful copulation; whereas humans and other domestic species are monovular, thereby normally only producing one live offspring per successful mating. This means there are very different mechanisms of control of follicle development in these species, especially after the development of the follicular antrum. After development of the antrum, in monovular species the follicles are competing for FSH stimulation, and there is a much smaller number of recruited follicles. Recruited follicles are distinguished by an increase in the expression of several steroidogenic enzymes. In monovular species there is the emergence of a so-called 'dominant' follicle which inhibits the other developing follicles in the recruited cohort (Webb et al, 2003). In contrast, it is possible that rather than inhibiting other follicles, in the pig the developing follicles may produce factors which enhance the rest of the growing follicles, to ensure many ovulations take place (Hunter et al, 1992). However, for the stages of preantral development it seems likely that while the follicles are at this very homogeneous stage of development, i.e. not yet competing for hormonal

stimulation that the mechanisms of control may be similar for monovular and polyovular species. Several factors have been shown to have similar effects in the culture of porcine preantral follicles and preantral follicles in monovular species such as the effect of FSH on follicle growth (pig: Hirao et al, 1994; sheep: Cecconi et al, 1999; cow: Itoh et al, 2002) and the anti-apoptotic effect of ascorbate on preantral follicle granulosa cell death (pig: current study; cow: Thomas et al, 2001; mouse: Murray et al, 2001). It would therefore seem likely that the mechanisms governing early follicle development may be similar in polyovular and monovular species, and the culture of early porcine follicles may help to elucidate stage-specific requirements for preantral follicle development in all species.

The results from the *in vitro* culture experiments carried out during the course of this study showed that porcine preantral follicles which are cultured in the presence of ascorbate display less atresia than those cultured under serum-free conditions in its absence. Ascorbate was therefore found to be a survival factor in this system, consistent with findings in other species (bovine: Thomas et al, 2001; mouse: Murray et al, 2001). FSH was found to promote follicle growth in this system, but it did not act as a survival factor as it had no effect on the death of somatic cells.

The characterisation of the requirements for preantral follicle development is extremely important in several respects. The development of a culture system capable of producing live young in the domestic species and humans is a distant reality, but if it is ever to be achieved it is vital that the requirements for folliculogenesis are characterised for every separate stage of development. If this is achieved then the production of live young through completely *in vitro*

matured oocytes could become a reality. The results from this experiment indicate that ascorbate is essential for early follicles, as it acts as a survival factor. The successful *in vitro* culture of porcine follicles therefore appears to depend on the addition of ascorbate to culture medium. FSH was shown to have actions on preantral follicles *in vitro*, as it increased follicle growth, but it did not act as a survival factor as it had no effect on cell death compared to controls. FSH may over-stimulate follicles at the preantral stage of development, so it seems possible it may be having a precocious effect.

The results from the present study also indicate that the culture of porcine preantral follicles in groups of three in 24-well plates is not beneficial to follicle survival in comparison to follicles cultured individually in 96-well plates. It was found by Wu et al (2001) that follicles survived best when cultured in groups of three in comparison to follicles cultured individually; however in the present study it was found that follicles displayed less granulosa cell death and also grew better when cultured in the absence of other follicles in 96-well plates. The identification of markers of follicle development is also important in the pursuit to develop a culture system for early follicles. If markers of development can be identified, for example secreted factors which are indicative of follicle/oocyte health or developmental stage, then a culture system could be changed and adapted to sustain the need of the particular follicle. For example, if a factor was secreted by a follicle which was particularly associated with antral cavity formation, and this factor was subsequently detected in culture medium, the culture conditions could be changed to sustain antral follicle development by perhaps increasing the gonadotrophin concentration of the medium. In the present experiments the potential of the matrix

metalloproteinases (MMPs) as markers of follicle development was examined. MMPs have been identified as markers of preantral follicle health in the cow. In this study MMP production was identified in culture medium, but was not found to be indicative of follicle health or any other parameter of follicle development. However, MMP-2 and MMP-9 were found to be secreted by porcine preantral follicles *in vitro*, and it is possible that more extensive experiments in the future could identify a possible role for the MMPs as markers of follicle development. MMP-2 and MMP-9 were also found to be secreted by denuded oocytes and cumulus oocyte complexes, although significantly more MMP-2 was secreted by the complexes, so it would appear that MMP-2 is probably a cumulus-secreted factor. There appeared to be more MMP-9 secreted by denuded oocytes than the cultured complexes, but this difference was not significant. This indicates that MMP-9 is probably an oocyte-secreted factor, but further work is required to confirm this.

Growth and differentiation factor-9 (GDF-9) is an oocyte-specific factor which had been identified in most species (human: Aaltonen et al, 1999; rodent: Jaatinen et al, 1999; sheep and cow: Bodensteiner et al, 1999) but not in the pig. Experiments carried out for this thesis revealed that porcine GDF-9 (isolated using human/mouse primers from Bodensteiner et al, 1999) is 88% homologous with the human sequence in the sequenced region of exon 2. Expression analysis using RT-PCR revealed that porcine GDF-9 appears to be expressed exclusively in the oocytes, as has been found in other species. GDF-9 β (BMP-15) is a closely related member of the TGF- β family, and is also oocyte specific (Dube et al, 1998). Using sheep primers for BMP-15 it was found using RT-PCR that in the pig BMP-15 also appears to be an oocyte-specific factor, although further

studies are required to confirm this. GDF-9 and BMP-15 could both be useful markers of follicle and oocyte development.

In conclusion the results from the experiments carried out during the course of this thesis indicate that ascorbate has an effect as a survival factor on porcine preantral follicles, and FSH affected the growth of follicles but did not have a positive effect on cell health. The production of MMP-2 and MMP-9 by cultured porcine preantral follicles, cumulus-oocyte complexes and denuded oocytes was demonstrated, but the presence of these enzymes in culture medium was not indicative of follicle health. Finally porcine GDF-9 was isolated from oocytes and it was shown that GDF-9 appears to be an oocyte-specific factor which displays a high degree of homology in comparison to other species in the sequenced region of exon 2. BMP-15 also appears to be oocyte-specific in the pig.

The findings from this thesis have hopefully led to a better understanding of early follicle development in the porcine, and this information may be useful in the ongoing quest to develop a long term culture system for the development of follicles in large mammals.

8 References

- Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O.J (1999). Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *J Clin Endocrinol Metab*; **84**: 2744-50
- Abeydeera LR, Wang WH, Prather RS, Day BN. (1998). Maturation in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro. *Biol Reprod*; **58**: 1316-20
- Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM. (2001). Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol*; **233**: 258-70
- Aktas H, Wheeler MB, Rosenkrans CF Jr, First NL, Leibfried-Rutledge ML. (1995). Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP]_i. *J Reprod Fertil*; **105**:227-235.
- Albrecht KH, Eicher EM. (2001). Evidence that *Sry* is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Dev Biol*; **240**: 92-107
- Amsterdam A, Koch Y, Lieberman ME, Lindner HR. (1975). Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. *J Cell Biol*; **67**: 894-900
- Anderson E, Albertini DF. (1976). Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J Cell Biol*; **71**: 680-6.

Andreu C, Parborell F, Vanzulli S, Chemes H, Tesone M. (1998). Regulation of follicular luteinization by a gonadotropin-releasing hormone agonist: relationship between steroidogenesis and apoptosis. *Mol Reprod Dev*; **51**: 287-94

Antonsson B, Martinou JC. (2000). The Bcl-2 protein family. *Exp Cell Res*; **256**: 50-7.

Armstrong DT, Goff AK, Dorrington JH. (1979). Regulation of follicular oestrogen synthesis In: *Ovarian Follicular Development and Function* edited by Midgley AR, Sadler WA. Raven Press, New York. P169-181.

Bagavandoss P. (1998). Differential distribution of gelatinases and tissue inhibitor of metalloproteinase-1 in the rat ovary. *J Endocrinol*; **158**: 221-8.

Baker SJ, Srsen V, Lapping R, Spears N. (2001). Combined effect of follicle-follicle interactions and declining follicle-stimulating hormone on murine follicle health in vitro. *Biol Reprod*; **65**: 1304-10.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS (1997). Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biol Reprod*; **56**: 1158–1168

Berne and Levy. (1996). *Principles of Physiology*. 2nd Edition.

Bielski BJH, Richter HW, Chan PC. (1975). Some properties of the ascorbate free radical. *Ann NY Acad Sci*; **258**: 231-238.

Black JL, Erickson BH. (1968). Oogenesis and ovarian development in the prenatal pig. *Anat Rec*; **161**: 45-55

Bodensteiner KJ, Clay CM, Moeller CL, Sawyer HR. (1999). Molecular cloning of the ovine Growth/Differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biol Reprod* **60**: 381-6

Bolamba D, Floyd AA, McGlone JJ, Lee VH. (2002). Epidermal growth factor enhances expression of connexin 43 protein in cultured porcine preantral follicles. *Biol Reprod*; **67**: 154-60.

Braw-Tal, R., Yossefi, S. (1997). Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil*; **109**: 165–171.

Briggs DA, Sharp DJ, Miller D, Gosden RG. (1999). Transferrin in the developing ovarian follicle: evidence for de-novo expression by granulosa cells. *Mol Hum Reprod*; **5**: 1107-1114

Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. (1990). FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol*; **138**: 16-25

Burgoyne PS, Buehr M, Koopman P, Rossant J, McLaren, A. (1988). Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XXXXY chimeric mouse testes. *Development*; **102**: 443-450.

Burns KH, Yan C, Kumar TR, Matzuk MM. (2001). Analysis of ovarian gene expression in follicle-stimulating hormone beta knockout mice. *Endocrinology*; **142**: 2742-51.

Byskov AG. (1974). Cell kinetic studies of follicular atresia in the mouse ovary. *J Reprod Fertil*; **37**: 277-85.

Campbell BK, Scaramuzzi RJ, Webb R. (1996). Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *J Reprod Fertil*; **106**: 7-16.

Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. (1998). Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol*; **204**: 373-84

Cardenas H, Pope WF. (2002). Androgen receptor and follicle-stimulating hormone receptor in the pig ovary during the follicular phase of the estrous cycle. *Mol Reprod Dev*; **62**: 92-8.

Cecconi S, Barboni B, Coccia M, Mattioli M. (1999). In vitro development of sheep preantral follicles. *Biol Reprod*; **60**: 594-601

Channing CP, Gordon WL, Liu W-K, Ward DW. (1985). The physiology and biochemistry of ovarian inhibin. *Proc Soc Exp Biol Med*; **178**: 339-61.

Chen W, Fu X, Sheng Z. (2002). Review of current progress in the structure and function of Smad proteins. *Chin Med J (Engl)*; **115**: 446-50

(a) Chesnel F, Eppig JJ. (1995). Synthesis and accumulation of p34cdc2 and cyclin B in mouse oocytes during acquisition of competence to resume meiosis. *Mol Reprod Dev*; **40**: 503-8.

(b) Chesnel F, Eppig JJ. (1995). Induction of precocious germinal vesicle breakdown (GVB) by GVB-incompetent mouse oocytes: possible role of mitogen-activated protein kinases rather than p34cdc2 kinase. *Biol Reprod*; **52**: 895-902.

Christmann L, Jung T, Moor RM. (1994). MPF components and meiotic competence in growing pig oocytes. *Mol Reprod Dev*; **38**: 85-90.

Chun SY, Billig H, Tilly JL, Furuta I, Tsafiriri A, Hsueh AJ. (1994). Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. *Endocrinology*; **135**: 1845-53.

Cortvrindt R, Smitz J, Van Steirteghem AC. (1997). Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture in vitro. *Hum Reprod*; **12**: 759-68.

Cortvrindt RG, Hu Y, Liu J, Smitz JE. (1998). Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin. *Fertil Steril*; **70**: 1114-25.

Coskun S, Uzumcu M, Lin YC, Friedman CI, Alak BM. (1995). Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterization of oocyte-produced factor(s). *Biol Reprod*; **53**: 670-5

Cupps PT. (1991). *Reproduction in Domestic Animals* 4th Edition. Academic Press.

Curry TE Jr, Mann JS, Estes RS, Jones PB. (1990). Alpha 2-macroglobulin and tissue inhibitor of metalloproteinases: collagenase inhibitors in human preovulatory ovaries. *Endocrinology*; **127**: 63-8

Curry Jr TE, Mann JS, Huang MH, Keeble SC . (1992). Gelatinase and proteoglycanase activity during the periovulatory period in the rat. *Biol Reprod*; **46**: 256-264

Curry TE Jr, Osteen KG. (2001). Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. *Biol Reprod*; **64**: 1285-96.

Dalin AM. (1987). Ovarian follicular activity during the luteal phase in gilts. *Zentralbl Veterinarmed A* ; **34**: 592-601

Deane HW. (1952). Histochemical observation on the ovary and oviduct of the albino rat during the estrus cycle. *Am J of Anatomy*; **91**: 363-413

de Jong G, van Dijk JP, van Eijk HG. (1990) The biology of transferrin. *Clin Chim Acta*; **190**: 1–46.

Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. (1998). Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A*; **95**: 13612-7.

Dissen GA, Romero C, Hirshfield AN, Ojeda SR. (2001). Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology*; **142**: 2078-86

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*; **383**: 531-5

Drury RAB, Wallington EA. (1976). Carleton's Histological Technique, 4th edn. Oxford University Press; 126-130

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. (1998). The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Mol Endocrinology*; **12**: 1809-17

Dufour J, Cahill LP, Mauleon P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *J Reprod Fertil*; **57**: 301-9.

Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. (1999). Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology*; **140**: 5789-96.

Durlinger AL, Grujters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. (2002). Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology*; **143**: 1076-84

Eckery DC, Moeller CL, Nett TM, Sawyer HR. (1997). Localization and quantification of binding sites for follicle-stimulating hormone, luteinizing hormone, growth hormone, and insulin-like growth factor I in sheep ovarian follicles. *Biol Reprod*; **57**: 507-13.

Edwards RG. (1965). Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*; **208**: 349-51

Edwards JG, McLaren J, Jones JL, Waller DA, O'Byrne KJ. (2003). Matrix metalloproteinases 2 and 9 (gelatinases A and B) expression in malignant mesothelioma and benign pleura. *Br J Cancer*; **88**: 1553-9.

Elvin JA, Yan C, Matzuk MM. (2000). Oocyte-expressed TGF-beta superfamily members in female fertility. *Mol Cell Endocrinol*; **159**: 1-5.

Endo T, Aten RF, Wang F, Behrman HR. (1993). Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology*; **133**: 690-8.

Eppig JJ, Schroeder AC. (1989). Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. *Biol Reprod*; **41**: 268-76

Eppig JJ. (1991). Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays*; **13**: 569-574.

Eppig JJ, Peters AH, Telfer EE, Wigglesworth K. (1993). Production of cumulus expansion enabling factor by mouse oocytes grown in vitro: preliminary characterization of the factor. *Mol Reprod Dev*; **34**: 450-6.

Eppig, JJ. The ovary: oogenesis, in: S.G. Hillier, H.C. Kitchener, J.P. Neilson (Eds.). (1996). *Scientific Essentials of Reproductive Medicine*, WB Saunders, London, UK, pp. 147–159.

Eppig JJ, O'Brien MJ. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod*; **54**: 197-207

Eppig JJ, Wigglesworth K, Pendola F, Hirao Y. (1997). Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod*; **56**: 976-84.

Eppig JJ, O'Brien MJ, Pendola FL, Watanabe S. (1998). Factors affecting the developmental competence of mouse oocytes grown in vitro: follicle-stimulating hormone and insulin. *Biol Reprod*; **59**: 1445-53.

Eppig JJ. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction*; **122**: 829-38.

Eppig JJ, Wigglesworth K, Pendola FL. (2002). The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci U S A*; **99**: 2890-4.

Erickson BH. (1967). Radioresponse of the pre-puberal porcine ovary. *Int J Radiat Biol Relat Stud Phys Chem Med*; **13**: 57-67.

Espey LL. (1980). Ovulation as an inflammatory reaction--a hypothesis. *Biol Reprod*; **22**: 73-106.

Espey LL, Coons PJ, Marsh JM, LeMaire WJ. (1981) Effect of indomethacin on preovulatory changes in the ultrastructure of rabbit Graafian follicles. *Endocrinology*; **108**: 1040-8.

- Faddy MJ, Telfer E, Gosden RG. (1987). The kinetics of pre-antral follicle development in ovaries of CBA/Ca mice during the first 14 weeks of life. *Cell Tissue Kinet*; **20**: 551-60.
- Findlay JK. (1993). An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol Reprod*; **48**: 15-23
- Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE. (1998). Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues. *Endocrinology*; **139**: 2571-8
- Fouladi Nashta AA, Waddington D, Campbell KH. (1998). Maintenance of bovine oocytes in meiotic arrest and subsequent development In vitro: A comparative evaluation of antral follicle culture with other methods. *Biol Reprod*; **59**: 255-262.
- Fowler P (2003). 2-cell 2-gonadotrophin lecture: <http://www.bms.abdn.ac.uk>
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet*; **25**: 279-83.
- Giudice LC. (2001). Insulin-like growth factor family in Graafian follicle development and function. *J Soc Gynecol Investig*; **8** (Suppl 1): S26-9.
- Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. (1997). Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol*; **74**: 111-22

Gore-Langton RE, Daniel SA. (1990). Follicle-stimulating hormone and estradiol regulate antrum-like reorganization of granulosa cells in rat preantral follicle cultures. *Biol Reprod*; **43**: 65-72.

Gore-Langton RE, Armstrong DT. (1994). Follicular Steroidogenesis and its Control. *The Physiology of Reproduction*. IInd Edition. Editors: Knobil E and Neill JD. Raven Press, New York; p571-627.

Gosden RG, Telfer EE. (1987). Number of follicles and oocytes in mammalian ovaries and their allometric relationships. *J of Zool*; **211**: 169-175

Gottsch ML, Van Kirk EA, Murdoch WJ. (2002). Role of matrix metalloproteinase 2 in the ovulatory folliculo-luteal transition of ewes. *Reproduction*; **124**: 347-52.

Greenwald GS, Roy SK. (1994). Follicular Development and its control. *The Physiology of Reproduction*. IInd Edition. Editors: Knobil E and Neill JD. Raven Press, New York; 629-724.

Griffiths HR, Lunec, J. (2001). Ascorbic acid in the 21st century – more than a simple antioxidant *Environ Toxicol Pharmacol*; **10**: 173-82.

Grollman AP, Lehninger AL. (1957). Enzymatic synthesis of L-ascorbic acid in different animal species. *Arch. Biochem. Biophys.* **69**; 458-467

Gross J, Lapiere CM. (1962). Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A*; **48**: 1014-22

Guarnaccia MM, Takami M, Jones EE, Preston SL, Behrman HR. (2000). Luteinizing hormone depletes ascorbic acid in preovulatory follicles. *Fertil Steril*; **74**: 959-63.

Guthrie HD, Grimes RW, Cooper BS, Hammond JM. (1995). Follicular atresia in pigs: measurement and physiology. *J Anim Sci*; **73**: 2834-44

- Guthrie HD, Garrett WM. (2001). Apoptosis during folliculogenesis in pigs. *Reprod Suppl*; **58**:17-29
- Gutierrez CG, Campbell BK, Webb R. (1997). Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biol Reprod*; **56**: 608-16.
- Gutierrez CG, Ralph JH, Telfer EE, Wilmot I, Webb R. (2000). Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. *Biol Reprod*; **62**: 1322-8
- Hagglund AC, Ny A, Leonardsson G, N. (1999). Regulation and localization of matrix metalloproteinases and tissue inhibitors of metalloproteinases the mouse ovary during gonadotropin-induced ovulation. *Endocrinology*; **140**: 4351-8
- Hardy K, Wright CS, Franks S, Winston RM. (2000). In vitro maturation of oocytes. *Br Med Bull*; **56**: 588-602
- Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ. (1999). Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology*; **140**: 1236-44.
- Heldin C.-H., Miyazono K, ten Dijke P. (1997) TGF- β signalling from cell membrane to nucleus through SMAD proteins. *Nature*; **390**: 465–471.
- Hillier SG. (2001). Gonadotropic control of ovarian follicular growth and development. *Mol Cell Endocrinol*; **179**: 39-46.
- Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S. (1994). In vitro growth and maturation of pig oocytes. *J Reprod Fertil*; **100**: 333-9

Hisaw FL. (1947). Development of the graafian follicle and ovulation. *Physiol Rev* **27**: 95–119.

Hoch-Ligeti C, Bourne GH. (1948). Changes in the concentration and histological distribution of ascorbic acid in ovaries, adrenals and livers of rats during oestrus cycles. *Br J Pathol*; **29**: 400–407

Hovatta O, Silye R, Abir R, Krausz T, Winston RML. (1997). Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod*; **12**: 1032–1036.

Hsu SY, Hsueh AJ. (2000). Tissue-specific Bcl-2 protein partners in apoptosis: An ovarian paradigm. *Physiol Rev*; **80**: 593-614.

Hsueh AJ, Billig H, Tsafiriri A (1994). Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr Rev*; **15**: 707-24.

Huang W, Suzuki K, Nagase H, Arumugam S, Van Doren SR, Brew K. (1996). Folding and characterization of the amino-terminal domain of human tissue inhibitor of metalloproteinases-1 (TIMP-1) expressed at high yield in *E. coli*. *FEBS Lett.*; **384**: 155-61.

Huang CT, Weitsman SR, Dykes BN, Magoffin DA. (2001). Stem cell factor and insulin-like growth factor-I stimulate luteinizing hormone-independent differentiation of rat ovarian theca cells. *Biol Reprod*; **64**: 451-6.

Hughes FM Jr, Gorospe WC. (1991). Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology*; **129**: 2415-22.

Hunter MG, Biggs C, Faillace LS, Picton HM. (1992). Current concepts of folliculogenesis in monovular and polyovular farm species. *J Reprod Fertil Suppl*; **45**: 21-38.

Itoh T, Kacchi M, Abe H, Sendai Y, Hoshi H. (2002). Growth, antrum formation, and estradiol production of bovine preantral follicles cultured in a serum-free medium. *Biol Reprod*; **67**:1099-105.

Jaatinen R, Laitinen MP, Vuojolainen K, Aaltonen J, Louhio H, Heikinheimo K, Lehtonen E, Ritvos O. (1999). Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B. *Mol Cell Endocrinol*; **156**: 189-93

Josso N, Rey R, Gonzalès J. (2003). Sexual Differentiation. www.Endotext.com: Pediatric Endocrinology; Chapter 7.

Juengel JL, Smith GW, Smith MF, Youngquist RS, Garverick HA. (1994). Pattern of protein production by bovine corpora lutea during luteolysis and characterization of expression of two major secretory products of regressing corpora lutea. *J Reprod Fertil*; **100**: 515-20

Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP, Ritvos O, McNatty KP. (2002). Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biol Reprod*; **67**: 1777-89.

Kaipia A, Hsueh AJ. (1997). Regulation of ovarian follicle atresia. *Annu Rev Physiol*; **59**: 349-63.

Kanai Y, Koopman P. (1999). Structural and functional characterization of the mouse Sox9 promoter: implications for campomelic dysplasia. *Hum Mol Genet*; **8**: 691-6.

Khandoker MA, Imai K, Takahashi T, Hashizume K. (2001). Role of gelatinase on follicular atresia in the bovine ovary. *Biol Reprod*; **65**: 726-32

Klein SA, Anderson GL, Kennedy AB, Bond SJ. (2002). The effects of a broad-spectrum matrix metalloproteinase inhibitor on characteristics of wound healing. *J Invest Surg*; **15**: 199-207.

Koopman, P, Gubbay, J, Vivian, N, Goodfellow, P, Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature*; **351**: 117-121

Kramer MM, Harman MT, Brill AK. (1933). Disturbances of reproduction and ovarian changes in the guinea-pig in relation to vitamin C deficiency. *Am J of Phys*; **106**: 611-622

Kumar TR, Wang Y, Lu N, Matzuk MM. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet*; **15**: 201-4

Levine M, Morita K. (1985). Ascorbic acid in endocrine systems. *Vitamins and Hormones*; **42**: 1-64.

Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R. (1986). A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem Biophys Res Commun*; **138**: 1129-37.

Liu J, Koenigsfeld AT, Cantley TC, Boyd CK, Kobayashi Y, Lucy MC. (2000) Growth and the initiation of steroidogenesis in porcine follicles are associated with unique patterns of gene expression for individual components of the ovarian insulin-like growth factor system. *Biol Reprod*; **63**: 942-52.

Luck MR, Jeyaseelan I, Scholes RA. (1995). Ascorbic acid and fertility. *Biol Reprod*; **52**: 262-6.

- Manikkam M, Calder MD, Salfen BE, Youngquist RS, Keisler DH, Garverick HA. (2001). Concentrations of steroids and expression of messenger RNA for steroidogenic enzymes and gonadotropin receptors in bovine ovarian follicles of first and second waves and changes in second wave follicles after pulsatile LH infusion. *Animal Reprod Sci*; **67**: 189-203
- Mao J, Wu G, Smith MF, McCauley TC, Cantley TC, Prather RS, Didion BA, Day BN. (2002). Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral follicular development and antrum formation in vitro. *Biol Reprod*; **67**: 1197-203
- Markstrom E, Svensson ECh, Shao R, Svanberg B, Billig H. (2002). Survival factors regulating ovarian apoptosis -- dependence on follicle differentiation. *Reproduction*; **123**: 23-30.
- Martin A. (2003). Antioxidant vitamins E and C and risk of Alzheimer's disease. *Nutr Rev*; **61**: 69-73.
- Mason AJ, Niall HD, Seeburg PH. (1986). Structure of two human ovarian inhibins. *Biochem Biophys Res Commun*; **135**: 957-64.
- Massague J. (1998). TGF-beta signal transduction. *Annu Rev Biochem*; **67**: 753-91
- Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. (2002). Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science*; **296**: 2178-80.
- Mayo KE, Cerelli GM, Spiess J, Rivier J, Rosenfeld MG, Evans RM, Vale W. (1986). Inhibin A-subunit cDNAs from porcine ovary and human placenta. *Proc Natl Acad Sci U S A*; **83**: 5849-53.

McCaffery FH, Leask R, Riley SC, Telfer EE. (2000). Culture of bovine preantral follicles in a serum-free system: markers for assessment of growth and development. *Biol Reprod*; **63**: 267-73

McCoard SA, Wise TH, Ford JJ. (2003). Germ cell development in Meishan and White Composite gilts. *Anim Reprod Sci*; **77**: 85-105.

McCullagh DR. (1932). Dual endocrine activity of the testis. *Science*; **76**: 19-20

(a) McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJ. (1997). Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology*; **138**: 2417-24.

(b) McGee EA, Perlas E, LaPolt PS, Tsafiriri A, Hsueh AJ. (1997). Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats. *Biol Reprod*; **57**: 990-8.

McGee EA, Chun SY, Lai S, He Y, Hsueh AJ. (1999). Keratinocyte growth factor promotes the survival, growth, and differentiation of preantral ovarian follicles. *Fertil Steril*; **71**: 732-8.

McGrath SA, Esquela AF, Lee SJ. (1995). Oocyte-specific expression of growth/differentiation factor-9. *Mol Endocrinol*; **9**: 131-6.

McPherron AC, Lee SJ. (1993). GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *J Biol Chem*; **268**: 3444-9.

Meduri G, Charnaux N, Driancourt MA, Combettes L, Granet P, Vannier B, Loosfelt H, Milgrom E (2002). Follicle-stimulating hormone receptors in oocytes? *J Clin Endocrinol Metab*; **87**: 2266-76.

- Mensink A, de Haan LH, Lakemond CM, Koelman CA, Koeman JH. (1995). Inhibition of gap junctional intercellular communication between primary human smooth muscle cells by tumor necrosis factor alpha. *Carcinogenesis*; **16**: 1063-7
- Mintz, B, Russell, ES. (1957). Gene-induced embryological modifications of primordial germ cells in the mouse. *J Exp Zool*; **134**: 207-230.
- Monget P, Fabre S, Mulsant P, Lecerf F, Elsen JM, Mazerbourg S, Pisselet C, Monniaux D. (2002). Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domest Anim Endocrinol*; **23**: 139-54.
- Monniaux D, Mariana JC, Gibson WR. (1984). Action of PMSG on follicular populations in the heifer. *J Reprod Fertil*; **70**: 243-53.
- Monniaux D, de Reviers MM. (1989). Quantitative autoradiographic study of FSH binding sites in prepubertal ovaries of three strains of rats. *J Reprod Fertil*.; **85**: 151-62.
- Moor RM, Dai Y. (2001). Maturation of pig oocytes in vivo and in vitro. *Reproduction Supplement*, **58**: 91-104.
- Morbeck DE, Esbenshade KL, Flowers WL, Britt JH. (1992). Kinetics of follicle growth in the prepubertal gilt. *Biol Reprod*; **47**: 485-91
- Motlik J, Fulka J. (1976). Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. *J Exp Zool*; **198**: 155-62.
- Motlik J, Crozet N, Fulka JJ. (1984). Meiotic competence in vitro of pig oocytes isolated from early antral follicles. *Reprod Fertil*; **72**: 323-8.
- Murray AA, Molinek MD, Baker SJ, Kojima FN, Smith MF, Hillier SG, Spears N. (2001). Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles in vitro. *Reproduction*; **121**: 89-96

Nagase H, Woessner JF Jr. (1999). Matrix Metalloproteinases. *J Biol Chem*; **274**: 21491-94

Nagyova E, Prochazka R, Vanderhyden BC. (1999). Oocyectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium. *Biol Reprod*; **61**: 569-74

Nayudu PL, Fehrenbach A, Kiesel P, Vitt UA, Pancharatna K, Osborn S. (2001). Progress toward understanding follicle development in vitro: appearances are not deceiving. *Arch Med Res*; **32**: 587-94.

Newton H, Picton H, Gosden RG. (1999). In vitro growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue. *J Reprod Fertil*; **115**: 141-50.

Nicosia SV, Evangelista I, Batta SK. (1975). Rabbit ovarian follicles. I. Isolation technique and characterization at different stages of development. *Biol Reprod*; **13**: 423-47

Nilsson E, Parrott JA, Skinner MK. (2001). Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol*; **175**: 123-30.

O'Brien MJ, Pendola JK, Eppig JJ. (2003). A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod*; **68**: 1682-6

Otsuka F, Shimasaki S. (2002). A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. *Endocrinology*; **143**: 4938-41.

Padayatty SJ, Levine M. (2001). New insights into the physiology and pharmacology of vitamin C. *Can Med Assoc J*; **164**: 353-5.

- Padh H, Aleo JJ. (1985). Stability of ascorbic acid in solution. *IRCS Med Sci* **13**:1028
- Padh H, Parborell F, Dain L, Tesone M. (1991). Vitamin C: newer insights into its biochemical functions. *Nutr Rev*; **49**: 65-70
- Page DC, Fisher EM, McGillivray B, Brown LG. (1990). Additional deletion in sex-determining region of human Y chromosome resolves paradox of X,t(Y;22) female. *Nature*; **346**: 279-81.
- Parborell F, Dain L, Tesone M. (2001). Gonadotropin-releasing hormone agonist affects rat ovarian follicle development by interfering with FSH and growth factors on the prevention of apoptosis. *Mol Reprod Dev*; **60**: 241-7
- Parrott JA, Skinner MK. (1999). Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology*; **140**: 4262-71
- Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, et al. (1991). Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell*; **67**: 437-47.
- Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T. (1991). Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology*; **129**: 3200-3207
- Pincus G, Enzman EV. (1935). The comparative behaviour of mammalian eggs in vivo and in vitro I The activation of ovarian eggs. *J Exp Med* **62**: 665-675
- Pinnell SR. (1985). Regulation of collagen biosynthesis by ascorbic acid: a review. *Yale J Biol Med*; **58**: 553-9.

- Prochazka R, Nagyova E, Rimkevicova Z, Nagai T, Kikuchi K, Motlik J. (1991). Lack of effect of oocyectomy on expansion of the porcine cumulus. *J Reprod Fertil*; **93**: 569-76
- Prunier A, Quesnel H. (2000). Influence of the nutritional status on ovarian development in female pigs. *Anim Reprod Sci*. **60-61**:185-97.
- Puistola U, Salo T, Martikainen H, Ronnberg L. (1986). Type IV collagenolytic activity in human preovulatory follicular fluid. *Fertil Steril*; **45**: 578-80.
- Quesnel H, Pasquier A, Jan N, Prunier A. (2000). Influence of insulin treatment and feed restriction on follicular development in cyclic gilts. *Anim Reprod Sci*; **64**: 77-87.
- Racowsky C, Baldwin KV. (1989). In vitro and in vivo studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrana/cumulus granulosa cell contact. *Dev Biol*; **134**: 297-306.
- Ralph JH, Telfer EE, Wilmut I. (1995). Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. *Mol Reprod Dev*; **42**: 248-53.
- Rannikki AS, Zhang FP, Huhtaniemi IT. (1995). Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Mol Cell Endocrinol*; **107**: 199-208
- Ratky J, Brussow KP, Solti L, Torner H, Sarlos P. (2001). Ovarian response, embryo recovery and results of embryo transfer in a Hungarian native pig breed. *Theriogenology*; **56**: 969-78.

Reissmann T, Felberbaum R, Diedrich K, Engel J, Comaru-Schally AM, Schally AV. (1995). Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: an overview. *Hum Reprod*; **10**: 1974-1981

Rideout WM 3rd, Eggan K, Jaenisch R. (2001). Nuclear cloning and epigenetic reprogramming of the genome. *Science*; **293**:1093-8

Riley SC, Leask R, Chard T, Wathen NC, Calder AA, Howe DC. (1999). Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases into the intrauterine compartments during early pregnancy. *Mol Hum Reprod*; **5**: 376-81.

Riley SC, Gibson AH, Leask R, Mauchline DJ, Pedersen HG, Watson ED. (2001). Secretion of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases into follicular fluid during follicle development in equine ovaries. *Reproduction*; **121**: 553-60.

Rivers JM, Devine MM. (1975). Relationships of ascorbic acid to pregnancy, and oral contraceptive steroids. *Ann N Y Acad Sci*; **258**: 465-82

Rivier C, Rivier J, Vale W. (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science*; **234**: 205-8.

Roy SK, Greenwald GS. (1985). An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. *Biol Reprod*; **32**: 203-15.

Roy SK, Greenwald GS. (1989). Hormonal requirements for the growth and differentiation of hamster preantral follicles in long-term culture. *J Reprod Fertil*; **87**: 103-14.

Russell DL, Salamonsen LA, Findlay JK. (1995). Immunization against the N-terminal peptide of the inhibin alpha 43-subunit (alpha N) disrupts tissue remodeling and the increase in matrix metalloproteinase-2 during ovulation. *Endocrinology*; **136**: 3657-64.

Salustri A, Yanagishita M, Hascall VC. (1990). Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. *Dev Biol*; **138**: 26-32.

Sanyal S, Datta S. (1979). Effect of ascorbic acid on in vitro rat adrenal & ovarian steroidogenesis. *Indian J Exp Biol*; **17**: 86-8.

Sasson R, Winder N, Kees S, Amsterdam A. (2002). Induction of apoptosis in granulosa cells by TNF alpha and its attenuation by glucocorticoids involve modulation of Bcl-2. *Biochem Biophys Res Commun*; **294**: 51-9

Shull MM, Doetschman T. (1994). Transforming growth factor-beta 1 in reproduction and development. *Mol Reprod Dev*; **39**: 239-46.

Shuttleworth G, Broughton Pipkin F, Hunter MG. (2002). In vitro development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II. *Reproduction*; **123**: 807-18

Sidis Y, Tortoriello DV, Holmes WE, Pan Y, Keutmann HT, Schneyer AL. (2002). Follistatin-related protein and follistatin differentially neutralize endogenous vs. exogenous activin. *Endocrinology*; **143**: 1613-24.

Sirard MA. (2001). Resumption of meiosis: Mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*; **55**:1241-54.

- Slomczynska M, Duda M, Slozak K. (2001). The expression of androgen receptor, cytochrome P450 aromatase and FSH receptor mRNA in the porcine ovary. *Folia Histochem Cytobiol*; **39**: 9-13
- Smith MF, Kemper CN, Smith GW, Goetz TL, Jarrell VL. (1994). Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells. *J Anim Sci*; **72**: 1004-12.
- Smith MF, McIntush EW, Ricke WA, Kojima FN, Smith GW. (1999). Regulation of ovarian extracellular matrix remodelling by metalloproteinases and their tissue inhibitors: effects on follicular development, ovulation and luteal function. *J Reprod Fertil Suppl*; **54**: 367-81.
- Sorensen RA, Wassarman PM. (1976). Relationship between growth and meiotic maturation of the mouse oocyte. *Dev Biol*; **50**: 531-536.
- Souza CJ, MacDougall C, MacDougall C, Campbell BK, McNeilly AS, Baird DT. (2001). The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type1 B (BMPRII) gene. *J Endocrinol*; **169**: R1-6.
- Soyal SM, Amleh A, Dean J. (2000). FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development*; **127**: 4645-54.
- Spears N, Boland NI, Murray AA, Gosden RG. (1994). Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. *Hum Reprod*; **9**: 527-32
- Spears N, de Bruin JP, Gosden RG. (1996). The establishment of follicular dominance in co-cultured mouse ovarian follicles. *J Reprod Fertil*; **106**: 1-6.
- Stansfield DA, Flint AP. (1967). The entry of ascorbic acid into the corpus luteum in vivo and in vitro and the effect of luteinizing hormone. *J Endocrinol*; **39**: 27-35

- Sternlicht MD, Werb Z. (2001). How Matrix Metalloproteinases Regulate Cell Behaviour. *Annu Rev Cell Dev Biol*; **17**: 463–516
- Strauss JF 3rd, Schuler LA, Rosenblum MF, Tanaka T. (1981). Cholesterol metabolism by ovarian tissue. *Adv Lipid Res*; **18**: 99-157.
- Sugano M, Shinogi T, Nakada K, Nakao T. (2001). Endocrine profiles and embryo quality in Japanese black cattle superovulated with human menopausal gonadotrophin and porcine follicle stimulating hormone. *Reprod Domest Anim*; **36**: 57-63.
- Sun GW, Kobayashi H, Suzuki M, Kanayama N, Terao T. (2003). Follicle-Stimulating Hormone and Insulin-Like Growth Factor I Synergistically Induce Up-Regulation of Cartilage Link Protein (Crtl1) via Activation of Phosphatidylinositol-Dependent Kinase/Akt in Rat Granulosa Cells. *Endocrinology*; **144**: 793-801
- Tadakuma H, Okamura H, Kitaoka M, Iyama K, Usuku G. (1993). Association of immunolocalization of matrix metalloproteinase 1 with ovulation in hCG-treated rabbit ovary. *J Reprod Fertil*; **98**: 503-8.
- Telfer EE. (1996). The development of methods for the isolation and culture of preantral follicles from bovine and porcine ovaries. *Theriogenology*; **45**: 101-110.
- Telfer EE, Binnie JP, McCaffery FH, Campbell BK. (2000). In vitro development of oocytes from porcine and bovine primary follicles. *Mol Cell Endocrinol*; **163**: 117-23.
- Themmen APN, Huhtaniemi IT. (2000). Mutations of Gonadotropins and Gonadotropin Receptors: Elucidating the Physiology and Pathophysiology of Pituitary-Gonadal Function. *Endocrine Rev*; **21**: 551-583.

Thomas FH, Leask R, Srsen V, Riley SC, Spears N, Telfer EE. (2001). Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture. *Reproduction*; **122**: 487-95.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ. (1991). Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology*; **129**: 2799-801.

(a) Tilly JL, LaPolt PS, Hsueh AJ. (1992). Hormonal regulation of follicle-stimulating hormone receptor messenger ribonucleic acid levels in cultured rat granulosa cells. *Endocrinology*; **130**: 1296-302.

(b) Tilly JL, Billig H, Kowalski KI, Hsueh AJ. (1992). Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. *Mol Endocrinol*; **6**: 1942-50.

Tilly JL, Tilly KI. (1995). Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology*; **136**: 242-252.

Tilly JL, Tilly KI, Kenton ML, Johnson AL. (1995). Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. *Endocrinology*; **136**: 232-41.

Tisdall DJ, Watanabe K, Hudson NL, Smith P, McNatty KP. (1995). FSH receptor gene expression during ovarian follicle development in sheep. *J Mol Endocrinol*; **15**: 273-81.

Torrance C, Telfer E, Gosden RG. (1989). Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture. *J Reprod Fertil*; **87**: 367-74.

Tsafiri A, Channing CP. (1975). Influence of follicular maturation and culture conditions on the meiosis of pig oocytes in vitro. *J Reprod Fertil*; **43**:149-52

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature*; **321**: 776-9.

Vale W, Rivier C, Hsueh A, Campen C, Meunier H, Bicsak T, Vaughan J, Corrigan A, Bardin W, Sawchenko P, et al. (1988). The chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog. Horm. Res*; **44**: 1-34.

Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. (1990). Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol*; **140**: 307-17

Vanderhyden BC, Telfer EE, Eppig JJ. (1992). Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles in vitro. *Biol Reprod*; **46**: 1196-204.

Vanderhyden BC . (1993). Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). *J Reprod Fertil*; **98**: 219-27

Vanderhyden BC, Tonary AM. (1995). Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by A factor(s) secreted by the oocyte. *Biol Reprod*; **53**:1243-50

Vigier B, Watrin F, Magre S, Tran D, Josso N. (1987). Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it in vitro. *Development*; **100**: 43-55

- Vitt UA, McGee EA, Hayashi M, Hsueh AJ. (2000). In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology*; **141**: 3814-20
- Wandji SA, Pelletier G, Sirard MA. (1992). Ontogeny and cellular localization of 125I-labeled insulin-like growth factor-I, 125I-labeled follicle-stimulating hormone, and 125I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. *Biol Reprod*; **47**: 814-22.
- Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. (1996). Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod*; **55**: 942-8
- Wandji, SA, Srsen, V, Nathanielsz, PW, Eppig, JJ, Fortune, JE. (1997). Initiation of growth of baboon primordial follicles in vitro. *Hum. Reprod*; **12**: 1993–2001.
- Webb R, Nicholas B, Gong JG, Campbell BK, Gutierrez CG, Garverick HA, Armstrong DG. (2003). Mechanisms regulating follicular development and selection of the dominant follicle. *Reprod Suppl* ;**61**: 71-90.
- Wickramasinghe D, Ebert KM, Albertini DF. (1991). Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev Biol*; **143**: 162-72.
- Wigley WC, Prihoda JS, Mowszowicz I, Mendonca BB, New MI, Wilson JD, Russell DW. (1994). Natural mutagenesis study of the human steroid 5 alpha-reductase 2 isozyme. *Biochemistry*; **33**: 1265-70.
- Woodruff TK, Mather JP. (1995). Inhibin, Activin and the Female Reproductive Axis. *Annu Rev Physiol*; **57**: 219-44
- Wright CS, Becker DL, Lin JS, Warner AE, Hardy K. (2001). Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation. *Reproduction*; **121**: 77-88.

Wright RJ, Holly JM, Galea R, Brincat M, Mason HD. (2002). Insulin-like growth factor (IGF)-independent effects of IGF binding protein-4 on human granulosa cell steroidogenesis. *Biol Reprod*; **67**: 776-81.

Wu J, Emery BR, Carrell DT. (2001). In vitro growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. *Biol Reprod*; **64**: 375-81

Yuan W, Lucy MC, Smith MF. (1996). Messenger ribonucleic acid for insulin-like growth factors-I and -II, insulin-like growth factor-binding protein-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. *Biol Reprod*; **55**: 1045-54.

Zhang J, Bai S, Zhang X, Nagase H, Sarras MP Jr. (2003). The expression of gelatinase A (MMP-2) is required for normal development of zebrafish embryos. *Dev Genes Evol*; **213**: 456-63.

Zhou J, Kumar TR, Matzuk MM, Bondy C. (1997). Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Mol Endocrinol*; **11**: 1924-33.

Web-pages:

The Smithsonian Institution- Human Origins programme.

<http://www.mnh.si.edu/anthro/humanorigins/index.htm>

LabQuest Biochemistry Unit Conversion

<http://www.vin.com/scripts/labquest/converthtml.pl>

Appendix 1

NCSU 23 medium (North Carolina State University)

Component	Mol wt	Conc. (mM)	g/1000ml	g/500ml
NaCl	58.45	108.73	6.3553	3.1776
NaHCO	84.00	25.07	2.1059	1.0529
KCl	74.55	4.78	0.3563	0.1782
KH ₂ PO ₄	136.09	1.19	0.1619	0.0810
MgSO ₄ ×7H ₂ O	246.50	1.19	0.2933	0.1467
CaCl ₂ ×2H ₂ O	147.00	1.70	0.2499	0.1250
Glucose	180.20	5.55	1.0001	0.5001
Glutamine	146.10	1.00	0.1461	0.0731
Taurine	125.10	7.00	0.8757	0.4379
Hypotaurine	109.10	5.00	0.5455	0.2728
Cysteine	157.60	1.00	0.1576	0.0788
Insulin		5 mg/l	0.0050	0.0025
Penicillin G	-	100iu/ml	(0.0650)	(0.0325)
Streptomycin	-	50mg/l	0.0500	0.0250

Appendix 2

SCOTT'S TAP WATER SUBSTITUTE Composition:

- Potassium bicarbonate 1g
- Magnesium sulphate 10g
- Distilled water 500ml

Appendix 3

The experiments performed in chapters 3 and 4 were carried out under very different conditions, in terms of supplements and conditions. The experiment described in chapter 3 was based on a successful culture system for bovine preantral follicles using McCoys serum-free medium (McCaffery et al, 2000; Thomas et al, 2001). The culture system described in chapter four was set up to try to examine the repeatability of a study carried out by Wu et al (2001), which involved higher concentrations of most added supplements and the basic medium used was NCSU 23. This system presumably provided far less physiological conditions in an attempt to push preantral follicle development more quickly than would be observed *in vivo*, to obtain fertilisable oocytes in a short period of time.

The differences in the supplements of the culture medium used in chapter 3 and 4 are likely to be profound. The high concentrations of supplements used in the Wu system are likely to have pushed development through quickly. The insulin concentration used in this system was increased 350-fold from the near physiological concentration of 10ng/ml used in the experiment in chapter 3. The information available on the effects of insulin on cultured porcine preantral follicles is limited; however studies that have been carried out on bovine granulosa cells suggest that increasing insulin concentration results in an increase in oestradiol output, and also an increase in the proliferative capacity of these cells. Dose response experiments have shown that proliferation and oestradiol output increased up to 5 μ g/ml in a study carried out by Gutierrez et al (1997). These results imply that the addition of insulin at 3.5 μ g/ml is likely to have accelerated development,

and this could help to explain how the Wu group (2001) obtained fertilisable oocytes from preantral follicles after just four days of culture.

The concentration of FSH which was used in chapters 3 and 4 also differed. The concentration used in chapter 3 was higher than that used in chapter 4. The concentration chosen for the McCoys experiment was selected as it has been found to be beneficial to cultured porcine preantral follicles (Hirao et al, 1994). It was used at a higher concentration than chapter 4 in the NCSU experiment at 2miu/ml (2miu/ml SIGMA porcine pituitary FSH is equivalent to $2\mu\text{g/ml}$ compared to 1.5ng/ml used by Wu et al {2001}). As FSH did not prove to be a survival factor in chapter 3 this suggests it may have pushed development too quickly. Wu et al (2001) carried out a dose response for FSH. From that study it appears that the optimal dose for FSH in porcine preantral follicles is 1.5ng/ml . If the experiment were to be repeated a dose response should also be carried out for the McCoys system to establish the optimum concentration for FSH using this system.

Transferrin is used in culture systems to transport essential iron from the media into the cell during cell culture. The iron is sequestered on the transferrin into the cell, and once transferred the protein absorbs free iron from the medium. Iron is a mineral which is essential, but also potentially harmful to cells. Increasing the concentration of transferrin to $10.5\mu\text{g/ml}$ from $2.5\mu\text{g/ml}$ is again likely to have accelerated cell processes. The concentration of transferrin found in human follicular fluid ranges from $0.93\mu\text{g/ml}$ to $2.79\mu\text{g/ml}$ (Briggs et al, 1999). As well as sequestering iron into cells it has also been postulated that transferrin can act as a

growth factor (de Jong et al, 1990). It therefore seems likely that the higher dose of transferrin would push development forward more quickly than the lower dose. Ascorbate concentration was doubled when comparing the experiment in chapter 3 with chapter 4. The ascorbate concentration chosen in chapter 3 (50 μ g/ml) was used as this dose was found to be beneficial in mouse (Murray et al, 2001) and the cow (Thomas et al, 2001). For the experiment described in chapter 4 the conditions were replicated from the Wu study (2001) which used a concentration of 100 μ g/ml. The study carried out by Murray et al (2001) culturing mouse preantral follicles, showed that when 28 μ g/ml and 280 μ g/ml of ascorbate were added to culture at the higher concentration follicles retained a significantly higher number of intact basement membranes after culture. This implies that 50 μ g/ml may not be a high enough concentration of ascorbate and 100 μ g/ml may be more appropriate, as was used in chapter 4.

The choice of serum also differed also between the experiments in chapters 3 and 4. In chapter 3 bovine foetal serum was used, and in chapter 4 porcine serum was used. It has been found that calf serum is more beneficial to porcine preantral follicles in previous studies by Telfer et al (unpublished), and since the present experiments have been carried out this has been confirmed by Mao et al (2002) where it was found that treatment with bovine serum produced a higher proportion of follicles which had developed an antrum after culture and there was a better recovery of cumulus-oocyte complexes than using porcine serum. This suggests bovine serum may provide the optimum conditions for porcine follicles.

In conclusion, the experiments from chapters 3 and 4 were very different in the supplements added, and also the basic medium used. In the McCoys experiment the concentrations of supplements were generally less, and this was used more as a model for developments *in vivo*. The NCSU co-culture experiment was carried out to try to emulate results obtained by Wu et al (2001), which is a system aimed to produce oocytes capable of fertilisation over a very short time-frame for preantral follicle development by providing medium which is unphysiological and drives development much more quickly than would be observed *in vivo*. It would be useful for future experiments to do dose-response experiments on these factors however, to determine the optimum concentration for use in porcine preantral follicle culture.